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Applicants : Linda S. Mansfield, Mary G. Rossano, Alice J. Murphy, and Ruth A. Vrable

Serial No. : 09/670,096 Confirmation No.: 7494

Filing Date : September 26, 2000

Title : VACCINE TO CONTROL EQUINE PROTOZOAL MYELOENCEPHALITIS IN HORSES

Group Art Unit : 1645

Examiner : Padmavathi Baskar, Ph.D.

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF UNDER 37 C.F.R. § 1.192

Sir:

This is an appeal from a final rejection in the above entitled application. The claims on appeal are set forth as Appendix A. An oral hearing will be requested. Enclosed are three (3) copies of this Brief and the fee due upon filing of the Brief.

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(1.) Real Party in Interest

The real party in interest is the Board of Trustees operating Michigan State University, East Lansing, Michigan, a constitutional corporation of the State of Michigan, which is the assignee of the above entitled application.

(2.) Related Appeals and Interferences

The present application is a divisional application of Application Serial No. 09/513,086 ('086), filed February 24, 2000, and which claims benefit of a provisional patent Application No. 60/152,193, filed September 2, 1999. The '086 application relates to a vaccine comprising the 16 and 30 kDa antigens. The present application is also related to Application Serial No. 09/670,355 ('355), relating to a vaccine comprising DNA encoding the 16 ±4 and 30 ±4 kDa antigens; Application Serial No. 09/669,833 ('833), which relates methods for making antibodies against the 16 ±4 and 30 ±4 kDa antigens; Application Serial No. 09/669,843 ('843) which relates to a monoclonal antibody which selectively binds to antigen; and Application Serial No. 09/670,244 ('224) which relates to recombinant protein comprising the 16 ±4 and 30 ±4 kDa

antigens. The above applications were all filed on September 26, 2000.

The '355 application and '843 are on appeal. The '086 application and '833 application are pending. The '244 application has been abandoned. No application has been allowed. There are no other related appeals and interferences.

(3.) Status of Claims

Claims 1, 2, and 21 are pending. No claims have been allowed.

(4.) Status of Amendments

An Amendment After Final was filed August 20, 2004.

(5.) Summary of Invention

As set forth in Claim 1, the applicants provide a composition for treating an equid infected with *Sarcocystis neurona* (Specification: page 15, lines 11-15) comprising a mixture of isolated antibodies against a 16 ±4 kDa antigen of *Sarcocystis neurona* and isolated antibodies against a 30 ±4 kDa antigen of *Sarcocystis neurona* (Specification: paragraph bridging pages 26-27) wherein the antibodies are from serum of an animal immunized with the antigen (Specification: page 26, lines 27-32; page 26, line 32, to page 27, line 22, and Example 1) and wherein the mixture is in a pharmaceutically acceptable carrier (Specification: page 14, lines 1-31).

As set forth in Claim 2, the applicants provide a further embodiment of the composition in which the antibodies are monoclonal antibodies (Specification: page 27, lines 4-22, and Example 1).

As set forth in Claim 21, the applicants also provide a method for treating an equid infected with *Sarcocystis neurona* (Specification: page 15, lines 11-15) comprising (a) providing a mixture of antibodies against a 16 ±4 kDa antigen and a 30 ±4 kDa antigen, both of which are specific to *Sarcocystis neurona*,

wherein the antibodies are selected from the group consisting of polyclonal antibodies (Specification: page 26, lines 27-32; page 26, line 32, to page 27, line 3) from serum from an animal immunized with the antigen (Specification: page 26, lines 27-32; page 26, line 32, to page 27, line 22, and Example 1), and monoclonal antibodies from a hybridoma (Specification: page 27, lines 4-22, and Example 1), and wherein the antibodies are in a pharmaceutically acceptable carrier (Specification: page 14, lines 1-31); and (b) inoculating the equid with the antibodies in the carrier to treat the equid (Specification: page 13, lines 24-35; page 15, lines 11-15).

(6.) Issues

(A) Claims 1, 2 and 21 remain rejected under 35 U.S.C. §112, first paragraph, as not being described in the specification in such a way so as to enable one skilled in the art to which it pertains or with which it is most nearly connected to make and/or use the invention.

(B) Claims 1, 2 and 21 remain rejected under 35 U.S.C. § 112, first paragraph, as not being enabled because specific monoclonal antibodies are required to

practice the claimed invention.

(C) Claim 2 was rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

(7.) Grouping of Claims

Claims 1 stands or falls on its own. Claim 21 and 2 each stands or falls on its own. Claim 21 is separately patentable since the method can utilize polyclonal antibodies from serum from an animal immunized with the antigen or monoclonal antibodies from a hybridoma.

(8.) Argument

(A.) Claims 1, 2 and 21 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled.

The applicants believe that the specification provides enablement which is commensurate with the scope of Claims 1, 2 and 21. When all of the evidence relating to the factors set forth in M.P.E.P. § 2164.01(a) for determining whether a disclosure satisfies the enablement requirement is considered, the evidence as a whole shows that the scope of the

applicants' claims are enabled by the applicants' disclosure.

The applicants' specification teaches that *Sarcocystis neurona* possesses a 16 ±4 and a 30 ±4 kDa antigen, both which are specific to *Sarcocystis neurona*, and which are useful for producing vaccines (Specification: page 13, lines 16-23). The applicants teach a variety of ways for vaccinating horses (Specification: page 13, lines 24-35), teach that the vaccines can comprise antibodies (Specification: page 15, lines 13-15), and teach how to make polyclonal antibodies (Specification: para. bridging pages 26-27) and monoclonal antibodies (Specification: page 27, lines 4-22; Example 1) for the vaccines. The applicants teach that antibody vaccines can be used for therapeutic treatment of horses infected with *Sarcocystis neurona* (Specification: page 15, lines 11-15) and to provide passive immunity (Specification: page 26, lines 20-26).

Liang (1998) provides an idea of what the state of the art was at about the time of the applicants' invention. Liang (1998) teaches that antisera from horses with EPM contain antibodies against 11, 14, 16, and 30 kDa antigens. Liang (1998) also teaches that *Sarcocystis neurona* "was sensitive to

specific antibodies but that "a 10-min exposure to antiserum was required to yield significant reduction in parasite production" (Liang (1998): page 1837). Liang (1998) further teaches that protective antibodies against some apicomplexan parasites may be effective *in vitro* but ineffective *in vivo* which Liang (1998) cites Hines *et al.* (Inf. Immun. 63: 349-352 (1995); copy enclosed) for support.

Hines showed that immunizing cattle with a vaccine containing the MSA-1 antigen of *Babesia bovis* failed to protect the immunized cattle against challenge with *Babesia bovis*. However, Hines suggested that an efficacious vaccine would include at least a second antigen of *Babesia bovis* as was shown in the case of a malaria vaccine which contained two antigens and which was shown to be effective *in vivo* against the malaria parasite whereas a vaccine containing only one of the antigens was partially effective (Hines: page 351, second para.). The applicants' claimed composition contains antibodies against a second antigen.

Liang (1998) also teaches that "[t]he high rate of exposure [of horses] to *Sarcocystis neurona* and the relatively low incidence of clinical EPM indicate that most horses develop effective immunity that may

prevent entry into the central nervous system [citing various sources]" (Liang (1998): page 1834, right col.). Liang (1998) also teaches that antisera from horses with EPM have antibodies against several *Sarcocystis neurona* antigens, in particular the 16 and 30 kDa antigens. Liang (1998) identifies the 11, 14, 16, and 30 as major immunogens (Liang (1998): Figure 1). Liang (1998) further teaches that antibodies against the 30 kDa antigen are not recognized as specific (Liang (1998): page 1837, left col., first para.), which suggests that the 30 kDa antigen is common to all *Sarcocystis* spp. and not unique to *Sarcocystis neurona*. However, the applicants teach that antisera from horses infected with *Sarcocystis neurona* contain antibodies specific for the 30 kDa antigen of *Sarcocystis neurona*. Therefore, since many horses exposed to *Sarcocystis neurona* do not have clinical signs of EPM but have immunity to *Sarcocystis neurona* the serum antibodies are likely effective for protecting against the parasite. The applicants teach that horses make antibodies specific to the 30 ±4 kDa antigen and that addition of CSF containing antibodies against both the 16 kDa and the 30 kDa antigens have a neutralizing effect upon merozoites of *Sarcocystis neurona* capacity to infect equine dermal cells (Table 1

on page 5 of the Declaration Under 37 C.F.R. § 1.132). Therefore a person of ordinary skill in the art would more likely than not believe that a composition comprising the 16 ±4 and 30 ±4 kDa antigens for treating horses infected with *Sarcocystis neurona* would be effective for treating the disease.

Liang (1998) provides support for a nexus between the 16 ±4 and 30 ±4 kDa-specific antibodies and their use in a composition for treating horses infected with *Sarcocystis neurona*. Liang (1998) teaches that the 14, 16, and 30 kDa antigens are surface antigens (Liang (1998): page 1836, left col., and Figure 3). Because surface antigens are generally important in the function or life-cycle of the organism, it is reasonable to expect that blocking the activity of one or more surface antigens by binding the antigens with antibodies would interrupt the function or life-cycle of the *Sarcocystis neurona*. Therefore, the applicants' presently claimed composition, which would bind to the 16 ±4 and 30 ±4 kDa antigens, would be expected to have at least some efficacy in treating horses infected with *Sarcocystis neurona*.

While Liang (1998) teaches that a "10-minute exposure to antiserum was required to yield a

significant reduction in parasite production" and that "may partially explain why protective antibodies to some apicomplexan parasites are effective in vitro but not in vivo" (Liang (1998): page 1837, left col., third para.), Liang (1998) suggests that the reason is that "newly released parasites are exposed to serum for a shorter time in vivo, and the access of neutralization-sensitive epitopes to antibody may be limited" and that "[m]erozoites in vivo may move more directly from cell to cell" (Liang (1998): page 1837, left col., third para.). While the statements suggest that humoral responses to *Sarcocystis neurona* may be of limited efficacy in inhibiting parasite production, the statements do not suggest that humoral responses would have no efficacy against disease caused by *Sarcocystis neurona*. In fact, Liang (1998) suggests that antibodies against the 14 and 16 kDa antigens may be efficacious against the EPM disease caused by *Sarcocystis neurona* because Liang (1998) also teaches that "in the case of EPM, disease occurs only after the merozoite passes through the vascular endothelium of the blood-brain barrier into the central nervous system, and so humoral responses may play an essential role in blocking this migration" (Liang (1998): page 1837, left col., third

para.) particularly since "specific cytotoxic T-cells are ineffective in attacking merozoites migrating to the central nervous system in the bloodstream" (Liang (1998): page 1837, left col., third para.). Liang (1998) further suggests that the 14 and 16 kDa antigens may be useful components of a subunit vaccine. Thus, the above suggests that the applicants' claimed composition comprising antibodies against the 16 ±4 and 30 ±4 kDa antigens might provide an efficacious means for treating horses infected with *Sarcocystis neurona*.

Therefore, in light of the above, the state of the art reasonably suggests that the applicants' claimed composition comprising a mixture of antibodies against several *Sarcocystis neurona* antigens would likely be efficacious for treating horses infected with *Sarcocystis neurona*. While the efficacy of a composition comprising a single antibody against a single antigen might be unpredictable, in light of the state of the art, one skilled in the art would likely believe that a composition comprising a mixture of antibodies against several antigens (16 ±4 and 30 ±4 kDa antigens) could provide an efficacious treatment for horses infected with *Sarcocystis neurona*.

Further, while the applicants do not provide

working examples showing that their claimed composition provides an efficacious treatment for horses infected with *Sarcocystis neurona*, the applicants' disclosure in light of the state of the art suggests that the applicants' claimed composition would be useful for treating horses infected with *Sarcocystis neurona*. The only experimentation expected would be adjusting the amounts of each antibody in the composition. Such experimentation is routine and could be performed by one skilled in the art without undue experimentation. According to 2164.02, the specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).

Furthermore, as taught in Liang, most horses exposed to *Sarcocystis neurona* do not develop clinical EPM which suggests that the horses have developed effective immunity which may have prevented entry of the parasite into the central nervous system (CNS). Thus, it appears that horses with EPM have some defect in their immune response which allowed the parasite to enter the CNS.

Liang also teaches that the immunodominant antigens are the 11, 14, 16, and 30 kDa antigens. Liang further provides data which suggests that while antibodies against the 14 and 16 kDa antigens are neutralizing, antibodies against the 30 kDa antigen are not neutralizing.

In contrast to the data in Liang, the applicants have data which show that at least some antibodies against the 30 kDa antigen are neutralizing. The data are presented in a Declaration under 37 C.F.R. § 1.132 (Appendix B) which clearly shows not only that some CSF antibodies against the 30 kDa antigen are neutralizing but that CSF containing antibodies against both the 16 and the 30 kDa antigens appeared to be more neutralizing than either antibody species alone. The applicants' data shows that a composition containing antibodies against both the 16 and 30 kDa antigens would be reasonably expected to provide an effective treatment for horses infected with *Sarcocystis neurona*. The applicants' data shows that if one skilled in the art had relied upon the teachings of Liang for guidance, they would have mistakenly believed that antibodies against the 30 kDa antigen were non-neutralizing.

It is important to note that in Liang, serum

and CSF samples were obtained from horses with a clinical diagnosis of a neurologic disorder resembling EPM. As taught by the applicants, horses with lameness or other neurologic diseases are being misdiagnosed as having EPM (page 4, lines 16-17). Since Liang does not demonstrate that the horses with clinical signs resembling EPM were actually infected with *Sarcocystis neurona* it is not known whether any of the Liang samples reported to contain only antibodies against a 30 kDa antigen (Liang: Figure 2) were infected with *Sarcocystis neurona*. The horses might have been infected with another *Sarcocystis* species which induces an antibody that reacts non-specifically with the 30 kDa antigen from *Sarcocystis neurona*. For example, the applicants show in U.S. Patent No. 6,153,394 to Mansfield *et al.*, which had been incorporated by reference on page 13, lines 16-20, that the 16 and 30 kDa antigens are *Sarcocystis neurona*-specific (Mansfield: col. 7, lines 37-46; Figure 4), that serum from horses known not to be infected with *Sarcocystis neurona* contain antibodies which in Western blots appear to cross-react with the 16 and 30 kDa antigens (Mansfield: col. 6, lines 43-50; Figure 3), and that the observed antibody cross-reactivity might be because

antibodies to other apicomplexian species can occur at or near the about 12 and 29 kDa bands and therefore may cause false-positive test results (Mansfield: col. 3, lines 11-19). Thus, Liang's support for the statement that antibodies against the 30 kDa antigen are not neutralizing is equivocal at best. Therefore, Liang is not believed to provide suitable support for the rejection.

Nevertheless, in light of the applicants' disclosure and declaration and other teachings in Liang, it would appear to be reasonable to believe that horses with EPM have an inadequate immune response to the parasite which is not sufficient to prevent entry of the parasite into the CNS and that boosting the immune response with antibodies against the 16 and 30 kDa antigens might provide a sufficient boost to an infected horse's immune response to inhibit entry of the parasite into the CSF. The applicants' currently claimed method provides such a means for treating such infected horses. The applicants' composition would boost the concentration of antibodies against the 16 and 30 kDa antigens in the horse. It would be reasonable to expect that the increased level of antibodies against those two antigens would have a beneficial effect on the horse

such as preventing further entry of the parasite into the CNS even if the increased level of antibodies did not cure the horse of the parasite.

Passive immunization of the presently claimed invention can be used for treating an equid that has not previously been exposed or developed an adequate immune response to *Sarcocystis neurona*. The equid thereby immediately acquires at least some protection to the parasite until it can mount an immune response, which may take several weeks. This treatment, even if not curing the horse, can improve the horse's resistance to *Sarcocystis neurona* at a critical early stage when the presence *Sarcocystis neurona* is first suspected. Passive immunization is often used in conjunction with other treatment regimens to improve outcomes. As can be seen in Table 1 on page 5 of the Declaration Under 37 C.F.R. § 1.132 the addition of CSF containing antibodies against both the 16 kDa and the 30 kDa antigens have a neutralizing effect upon merozoites of *Sarcocystis neurona* capacity to infect equine dermal cells. This data would lead a person of ordinary skill in the art to conclude that the claimed composition and method is likely to be beneficial to an equid infected with *Sarcocystis neurona*. The ability to neutralize

merozoite capacity to infect equine cells, even if incomplete, may slow progession of the disease until a more vigorous immune response can develop in the horse. Therefore the composition and method can be an effective treatment by delaying progession of the EPM and lessening severity of the disease.

Attached is an unpublished manuscript by Elsheikha *et al.* titled "*Monoclonal IgG antibody-mediated protection against Sarcocystis neurona infection*" which is a study done in mice that are widely used as a model for pathogenesis and infectivity studies of *Sarcocystis neurona*. In the experiments, mice treated with a mixture of monoclonal antibodies which reacted largely against an antigenic fraction with a molecular weight of 30 kDa developed clinical signs later than untreated mice. The mixture of monoclonal antibodies delayed the onset and severity of infection in treated mice compared to controls. While the monoclonal antibodies only slightly reacted against a 16 kDa antigen, this manuscript suggest to a person of ordinary skill in the art that a composition and method of treatment using antibodies against both a 16 kDa and a 30 kDa antigen would be effective in delaying and lessening severity of the *Sarcocystis neurona* infections.

Therefore, in light of the above, Claims 1, 2 and 21 are believed to be enabled by the applicants' disclosure.

(B.) Claims 1, 2 and 21 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement since specific monoclonal antibodies are required to practice the claimed invention. As required elements, they must be known and readily available to the public or obtainable by a repeatable method set forth in the specification. If they are not so obtainable or available, the enablement requirements may be satisfied by a deposit of the pertinent cells lines / hybridomas which produce these antibodies.

Applicants have deposited with the American Type Culture Collection (ATCC) a strain of *Sarcocystis neurona* so as to meet the requirements of the Budapest Treaty. It is believed that this deposit satisfies the requirements of 35 U.S.C. §112, first paragraph, since the monoclonal antibodies required to practice the invention are obtainable by a repeatable method set forth in the specification. According to M.P.E.P. 2164.01(a), there are many factors to be considered when

determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is undue. These factors include, but are not limited to: the breadth of the claims; the nature of the invention; the state of the prior art; the level of one of ordinary skill; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). In *In re Wands*, the court reversed the rejection for lack of enablement under 35 U.S.C. §112, first paragraph, concluding that undue experimentation would not be required to practice the invention. The nature of monoclonal antibody technology is such that experiments first involve the attempt to make monoclonal hybridomas to determine which ones secrete antibody with the desired characteristics. The court found that all of the methods needed to practice the invention were well known, and that there was a high level of skill in the art at the time the application was filed related to this technology (M.P.E.P.

2164.06(b)).

Considering the nature of the invention, the state of the prior art, the level of one of ordinary skill, the level of predictability in the art of producing polyclonal and monoclonal antibodies, and the amount of direction in the specification, polyclonal or monoclonal antibodies directed against the 16 ±4 kDa antigen and 30 ±4 kDa antigen of *Sarcocystis neurona* are obtainable by a person of ordinary skill in the art. Methods of producing hybridomas which produce monoclonal antibodies directed against the 16 ±4 kDa antigen and 30 ±4 kDa antigen of *Sarcocystis neurona* are described in Example 1 in the specification. Briefly, *Sarcocystis neurona* can be cultured on equine cell line cultures, the merozoites can be harvested, and the antigens can be purified by polyacrylamide gel electrophoresis by known methods. Hybridomas which provide monoclonal antibodies directed against the antigens can then be made by methods set forth in Example 1. Alternatively, polyclonal antibodies against the purified antigens can be isolated from immunized animals by methods well known in the art. Therefore, the specification provides sufficient enablement to a person of ordinary skill in the art to make the invention.

Applicants have deposited with the American Type Culture Collection (ATCC) a strain of *Sarcocystis neurona* so as to meet the requirements of the Budapest Treaty. *Sarcocystis neurona* can be cultured long term on equine dermal cells (available from ATCC, Manassas, Virginia) as described in Example 3 (Page 39, lines 20-34 in the specification). As noted above, polyclonal or monoclonal antibodies directed against the 16 ±4 kDa antigen and 30 ±4 kDa antigen of *Sarcocystis neurona* are obtainable by a person of ordinary skill in the art without undue experimentation by methods known in the art and described in the specification. Methods of producing hybridomas which produce monoclonal antibodies directed against the 16 ±4 kDa antigen and 30 ±4 kDa antigen of *Sarcocystis neurona* are described in Example 1 in the specification. Considering the level of one of ordinary skill in the art, the amount of direction provided by the inventor, and the quantity of experimentation needed to make polyclonal or monoclonal antibodies, one skilled in the art would be enabled to make the antibodies required to practice the claimed invention utilizing the teachings of the specification and the *Sarcocystis neurona* deposited with the ATCC.

(C.) Claim 2 was rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 2 has been amended in the Amendment Under 37 C.F.R. § 1.116 filed August 20, 2004 to improve clarity and resolve the issue of antecedent basis. The applicants are willing to amend the claim as required by the Board.

(9.) Conclusion

As shown above, Claims 1, 2 and 21 are enabled by the specification. Remand to the Examiner for Notice of Allowance is requested.

Respectfully,

Ian C. McLeod
Registration No. 20,931

MCLEOD & MOYNE, P.C.
2190 Commons Parkway
Okemos, MI 48864

(517) 347-4100
Fax:(517) 347-4103

Attachments:

Appendix A - Claims on Appeal

Appendix B - Declaration under 37 CFR 1.132

Appendix C -
Liang *et al. Inf. Immun.* 66(5): 1834-1838(1998).

Hines *et al. Inf. Immun.* 63(1): 349-352 (1995).

Appendix D -
Elsheikha *et al. "Monoclonal IgG antibody-mediated protection against Sarcocystis neurona infection".*

APPENDIX A

-1-

A composition for treating an equid infected with *Sarcocystis neurona* comprising a mixture of isolated antibodies against a 16 ±4 kDa antigen of *Sarcocystis neurona* and isolated antibodies against a 30 ±4 kDa antigen of *Sarcocystis neurona* wherein the antibodies are from serum of an animal immunized with the antigen and wherein the mixture is in a pharmaceutically acceptable carrier.

-2-

The composition of Claim 21 wherein the antibodies are monoclonal antibodies.

A method for treating an equid infected with *Sarcocystis neurona* comprising:

(a) providing a mixture antibodies against a 16 ±4 kDa antigen and a 30 ±4 kDa antigen, both of which are specific to *Sarcocystis neurona*, wherein the antibodies are selected from the group consisting of polyclonal antibodies from serum from an animal immunized with the antigen and monoclonal antibodies from a hybridoma, and wherein the antibodies are in a pharmaceutically acceptable carrier; and

(b) inoculating the equid with the antibodies in the carrier to treat the equid.

APPENDIX B

MSU 4.1-526
Appl. No. 09/670,096
April 1, 2003
Reply to Office Action of Jan. 23, 2003

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Linda S. Mansfield, Mary G. Rossano, Alice J. Murphy, and Ruth A. Vrable

Serial No. 09/670,096 Group Art Unit: 1645

Filing Date: 2000 September 26

Title: VACCINE TO CONTROL EQUINE PROTOZOAL MYELOENCEPHALITIS IN HORSES

Examiner: Padmavathi Basker, Ph.D.

BOX AF

Commissioner of Patents and Trademarks

Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132

Dear sir:

Alice J. Murphy states as follows.

(1) That she is an inventor of the invention in the above entitled application.

(2) That she performed an experiment in East Lansing, Michigan at Michigan State University (assignee of the present invention) to determine the neutralizing ability of antibodies against the 16 and 30 kDa antigens. The results showed that cerebral spinal fluid (CSF) from horses infected with *Sarcocystis neurona* which contained only antibodies that were strongly reacting against the 30 kDa antigen was neutralizing as was CSF which contained only antibodies that were strongly reacting against the 16 kDa antigen.

(3) That the experiment used CSF samples isolated from three horses infected with *Sarcocystis neurona*. CSF from the first infected horse contained antibodies which strongly

reacted against both the 16 kDa and 30 kDa antigens, CSF from the second infected horse contained antibodies which strongly reacted against only the 30 kDa antigen, and CFS from the third infected horse contained antibodies which strongly reacted against only the 16 kDa antigen. The controls for the experiment consisted of CSF from a horse from India known not to be infected with *Sarcocystis neurona* and Tris-buffered saline (TBS) containing 5% fetal bovine serum (FBS). The first horse was also culture positive for *Sarcocystis neurona*. Neural tissue from the horse at necropsy was ground up and inoculated into the media on equine dermal cells in culture. The media was replaced after 24, 48, and possibly 72 hours post inoculation and then weekly thereafter. The first plaque was seen on day 29 after inoculation. The merozoites from the plaques were subsequently identified as *Sarcocystis neurona* by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) techniques. Horses 2 and 3 had clinical signs which suggested the horses were infected with *Sarcocystis neurona*.

(4) That the experiment was performed as follows.

(a) Merozoites of *Sarcocystis neurona* from culture were washed in Tris-buffered saline (TBS) twice to remove media. The merozoites had been previously obtained from neural tissue from a Michigan horse infected with *Sarcocystis neurona*. The identity of the merozoites had been confirmed by PCR and RFLP.

(b) The washed merozoites were diluted in TBS and 35 μL was added to each of the 6 tubes comprising each of the horse groups. To test viability of the merozoites, 35 μL of the merozoites were affixed to a slide by cytospin (two replicates) and stained. There appeared to be about 20 to 30 viable merozoites per 35 μL. The stained cytospin provided an idea of the number of normal appearing and potentially viable merozoites per 35 μL aliquot. To confirm the viability of the merozoites, a real viability test was performed as follows. 70 μL of the merozoites were added directly to a 25 mL flask of confluent equine dermal cells. An additional 70 μL of merozoites were washed and spun twice in the same manner as the test samples. The pellet was

suspended in media and divided between two 25 mL flasks of confluent equine dermal cells. Two hours after inoculating the flasks, moving (spinning as they do when they are "drilling" into a cell) merozoites were seen in all three flasks. In addition, all three flasks developed plaques. Plaques consisted of a minimum of three rounded-up cells contiguous with one another to a maximum of a bare area of surface surrounded by rounded-up cells. (Cells round up when infected and come loose off the well or flask when the cell is heavily laden with parasite or the cell bursts from the parasite load. Since infective merozoites tend to move only into neighboring cells (unless one shakes up the flask which happens when the media is changed), bare areas surrounded by rounded up cells in older plaques are seen). The test for real viability confirmed that the merozoites used in the experiment described herein were viable.

(c) The CSF sample from horse 1 was diluted 1:10, 1:20, 1:40, 1:80, 1:160 with TBS and the ~~CFS~~ CSF samples from horses 2 and 3 were diluted 1:10, 1:20, 1:40, 1:80 with TBS. 200 μL of undiluted CSF and each dilution of CSF was each added to a tube of merozoites. The controls consisted of undiluted Indian horse CSF and TBS containing 5% FBS. There were six replicates of each of the samples and controls.

(d) All the samples and controls were incubated for one hour at 37° C.

(e) Each tube was centrifuged for 4 minutes at 1000 xg to pellet the merozoites. The supernatant fraction was removed and the merozoites were washed by resuspending the merozoite pellets in 300 μL TBS and centrifuging to pellet the merozoites and removing the supernatant fraction. Two washes were performed.

(f) After the final wash, the merozoite pellets were each resuspended in 200 μL of media and then each suspension was added to a well of a six–well plate of a monolayer of equine dermal cells which was just confluent.

(g) The plates were gently swirled to distribute the merozoites over the

monolayer and the cells then incubated at 37° C in a 5% CO_2 atmosphere. The media was replaced after 24 hours and then replaced weekly thereafter.

(h) Any plaques which formed were counted at five and six weeks post inoculation.

(5) That the results of the experiment are shown in Table 1; that the results show that the CSF containing antibodies against either antigen was separately neutralizing when used undiluted compared to the controls; that the results further show that CSF containing both antibodies was neutralizing even when used at a 1:10 dilution; and, that the results show that the neutralizing ability of the undiluted CSF from all three infected horses appears to be significant as was the 1:10 dilution of the CSF from the first horse.

Table 1

Sample	Dilution	Mean No. Plaques (SE) at 5 weeks post inoculation	Mean No. Plaques (SE) at 6 weeks post inoculation
1	undiluted	**4.3 (0.5)**	**20.0 (1.2)**
(Anti-16 & -30)	1:10	**3.2 (0.6)**	**30.0 (1.8)**
	1:20	6.0 (0.6)	55.5 (2.7)
	1:40	7.2 (0.4)	54.2 (2.8)
	1:80	9.0 (0.6)	52.7 (1.7)
	1:160	8.8 (0.4)	54.5 (2.3)
2	undiluted	**3.7 (0.4)**	**37.5 (2.4)**
(Anti-30)	1:10	7.8 (0.3)	57.5 (2.5)
	1:20	6.8 (0.3)	53.2 (3.0)
	1:40	8.3 (0.8)	53.2 (1.7)
	1:80	9.8 (0.4)	57.8 (3.1)
3	undiluted	**4.0 (0.6)**	**36.3 (2.1)**
(Anti-16)	1:10	7.7 (0.8)	55.3 (2.5)
	1:20	8.7 (0.6)	58.3 (3.1)
	1:40	8.7 (0.9)	55.5 (2.6)
	1:80	7.7 (0.8)	49.2 (2.4)
Indian horse	undiluted	8.7 (0.4)	56.8 (2.7)
5% FBS	undiluted	8.5 (0.6)	54.8 (3.2)

(6) That the results of Liang *et al.*, published in Infection and Immunity 66: 1834-1838 (1998), which shows that antibodies against the 30 kDa antigen in serum or CSF from horses infected with *Sarcocystis neurona* are not neutralizing, are not consistent with the results described herein and are not believed to be correct.

(7) That the undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Evidence that Surface Proteins Sn14 and Sn16 of *Sarcocystis neurona* Merozoites Are Involved in Infection and Immunity†

FANG TING LIANG, DAVID E. GRANSTROM, XIAO MIN ZHAO, AND JOHN F. TIMONEY*

Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546

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***Sarcocystis neurona* is the etiologic agent of equine protozoal myeloencephalitis (EPM). Based on an analysis of 25,000 equine serum and cerebrospinal fluid (CSF) samples, including samples from horses with neurologic signs typical of EPM or with histologically or parasitologically confirmed EPM, four major immunoblot band patterns have been identified. Twenty-three serum and CSF samples representing each of the four immunoblot patterns were selected from 220 samples from horses with neurologic signs resembling EPM and examined for inhibitory effects on the infectivity of *S. neurona* by an in vitro neutralization assay. A high correlation between immunoblot band pattern and neutralizing activity was detected. Two proteins, Sn14 and Sn16 (14 and 16 kDa, respectively), appeared to be important for in vitro infection. A combination of the results of surface protein labeling, immunoprecipitation, Western blotting, and trypsin digestion suggests that these molecules are surface proteins and may be useful components of a vaccine against *S. neurona* infection. Although *S. neurona* is an obligate intracellular parasite, it is potentially a target for specific antibodies which may lyse merozoites via complement or inhibit their attachment and penetration to host cells.**

The apicomplexan *Sarcocystis neurona* is the causative agent of equine protozoal myeloencephalitis (14), a progressive disease affecting the central nervous system (4, 7, 13). Cases of equine protozoal myeloencephalitis (EPM) have been reported among native horses in North, Central, and South America (3, 10, 11, 15, 16, 26). Serological testing based on immunoblot patterns in Kentucky, Ohio, Pennsylvania, and Oregon detected an average *S. neurona* exposure rate of 45% (5, 6, 18, 32). The New York State Veterinary College at Cornell University reported that 25% of equine neurologic disease accessions were due to EPM in 1978 (19). The number of cases diagnosed at necropsy at the Livestock Disease Diagnostic Center at the University of Kentucky increased from approximately 8% of all neurological accessions during 1988 to 1990 to 15% during 1991 to 1992 (19).

Although no successful vaccine against related apicomplexan parasites has been widely used, there are encouraging signs that such a vaccine is possible. Surface antigens of coccidia have been shown to be involved in interactions with the host cell membrane during invasion (9, 24), and apical complex proteins of some coccidia have been found to be targets of protective antibodies (24, 28, 33, 34). Apical complex organelles of sporozoites appear to secrete their contents during host cell attachment and formation of the parasitophorus vacuole (2, 30, 35).

Although the pathogenesis of EPM is not fully known, the following events are believed to occur. *S. neurona* sporozoites penetrate the horse's intestinal tract, enter vascular endothelial cells, and complete at least one merogonous generation. As immune responses, including antibody production are induced, merozoites may pass through the vascular endothelium of the blood-brain barrier into the immune privileged central nervous system, where they survive. The high rate of exposure to *S. neurona* and the relatively low incidence of clinical EPM indicate that most horses develop effective immunity that may prevent entry into the central nervous system (5, 6, 18, 32).

Since 1991, approximately 25,000 equine serum and cerebrospinal fluid (CSF) samples, including samples from horses with neurologic signs and with histologically or parasitologically confirmed EPM, have been tested for specific antibody to *S. neurona* at the University of Kentucky. Four immunoblot band patterns could be consistently identified in these samples. The objective of this study was to attempt to correlate immunoblot band patterns with in vitro neutralizing activity of the serum and CSF. Twenty-three serum and CSF samples, each from a different horse and representative of each of the four band patterns, were selected from a set of samples from 220 horses with a clinical diagnosis of a neurologic disorder resembling EPM and tested for inhibitory activities on parasite infection by an in vitro neutralization assay. Antibodies to two surface polypeptides were correlated with in vitro neutralizing activity.

MATERIALS AND METHODS

Parasite. *S. neurona* SN3 was originally isolated from the spinal cord of a horse with histologically confirmed EPM (16).

Cell and tissue culture medium. Bovine turbinate (BT) cells were purchased from the American Type Culture Collection (Rockville, Md.). Cells were seeded in 75- or 25-cm^2 tissue culture flasks (Corning Inc., Corning, N.Y.) and incubated in an atmosphere containing 5% CO_2 and 95% air at 37°C. The cell culture was maintained in RPMI 1640 supplemented with 15% fetal calf serum (FCS), 2 mM sodium pyruvate, 0.075% (wt/vol) sodium bicarbonate, 120 U of penicillin per ml, and 120 μg of streptomycin (BioWhittaker, Walkersville, Md.) per ml. Subconfluent cell culture was used in all of the experiments.

Clinical samples of serum and CSF. Twenty-three serum and CSF samples from different horses were selected to represent each immunoblot pattern from a group of samples from 220 horses with a clinical diagnosis of a neurologic disorder resembling EPM. These samples were originally submitted to our laboratory for serological testing for EPM from throughout the United States.

Immunoblotting. Immunoblotting was performed as previously described (17). Approximately 1.5 × 10^7 *S. neurona* merozoites were harvested from BT cell culture and dissolved in an appropriate volume of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (65 mM Tris, 2% SDS, 10% glycerol, 1.5% 2-mercaptoethanol [pH 6.8]). After heating in a boiling water bath for 5 min, the sample was separated in an SDS–10 to 20% linear gradient

* Corresponding author. Mailing address: Department of Veterinary Science, 108 Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099. Phone: (606) 257-4172. Fax: (606) 257-6169. E-mail: jtimoney@pop.uky.edu.

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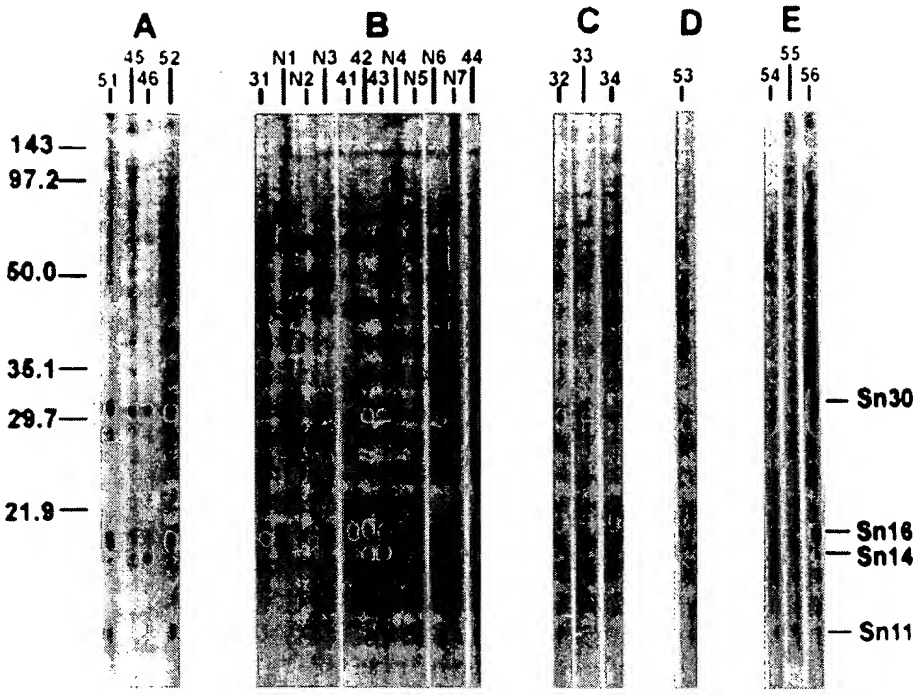

FIG. 1. Immunoblot patterns of proteins of *S. neurona* following reaction with equine serum and CSF samples. The five panels represent samples selected from each of five immunoblots of 45 serum and CSF samples performed in a Miniblotter 45. N4, N5, 34, and 51 were CSF samples. The numbers at the left are molecular masses (in kilodaltons).

polyacrylamide gel with a thickness of 0.75 mm, using a discontinuous buffer system (25). Separated proteins were electrotransferred to nitrocellulose (NC; Costar Co., Cambridge, Mass.) in Towbin transfer buffer (37). The blot was blocked in 5% nonfat dry milk and 0.4% Tween 20 in phosphate-buffered saline (PBS; pH, 7.2) and then placed in a Miniblotter 45 (Immunetics, Cambridge, Mass.). Serum or CSF diluted 1:10 or 1:2, respectively, with blocking solution, was applied. Biotinylated goat anti-horse immunoglobulin G, followed by streptavidin-peroxidase conjugate (Pierce, Rockford, Ill.) and aminoethyl carbazole-hydrogen peroxide, was used to develop the blot.

Neutralization assay. Serum and CSF samples were filtered through 0.22-μm-pore-size syringe filters (Micron Separations Inc., Westborough, Mass.). FCS was used as a control. Approximately 3.3×10^6 *S. neurona* merozoites freshly isolated from BT cell culture were resuspended in 1.0 ml of serum or CSF and incubated at 37°C for 60 min with occasional shaking. The merozoites were then pelleted by centrifugation at 300 × *g* for 5 min at 37°C. Each of the treated merozoite samples was seeded into three 25-cm^2 tissue culture flasks. Two days postinfection, merozoites remaining in the medium were removed and fresh medium was added. On day 5, schizonts in 10 fields (400×) of each flask were counted under a phase-contrast microscope. Merozoites were recovered and counted in a hemacytometer on day 7.

Surface protein labeling. About 8×10^7 merozoites freshly harvested from BT culture were washed twice with excess volumes of Na_2CO_3 buffer (50 mM Na_2CO_3, 0.85% NaCl [pH 7.4]) by centrifugation at 300 × *g* for 10 min at 37°C. The organisms were then resuspended in 1 ml of Na_2CO_3 buffer containing 100 μg ImmunoPure Sulfo-NHS-Biotin (Pierce), incubated at 37°C for 10 min, and then washed twice in excess volumes of Na_2CO_3 buffer at 4°C. The biotin-labeled merozoites were lysed in 1 ml of lysis buffer (50 mM Tris, 1% Triton X-100, 0.1% SDS, 1 mM EDTA [pH 7.6]) at 4°C for 30 min. The lysate was collected by centrifugation at 3,000 × *g* for 30 min at 4°C.

Immunoprecipitation. Preparations for immunoprecipitation were conducted at 4°C. Aliquots of lysate from 8×10^7 biotin-labeled merozoites were mixed with 150 μl of positive serum from horse with a histologically diagnosed case of EPM and with serum from a normal horse and incubated for 30 min. The mixtures were each incubated with 150 μl of GammaBind Plus Sepharose gel (Pharmacia LKB Biotechnology, Uppsala, Sweden) for an additional 30 min. The gels were washed twice with excess volumes of lysis buffer and once with PBS-Tween 20 (0.4% Tween 20 in PBS [pH 7.4]). Finally, the gels were mixed with 200 μl of SDS-PAGE sample buffer, heated in a boiling water bath for 5 min, and centrifuged at 3,000 × *g* for 30 min.

Western blotting. Approximately 3×10^5 biotin-labeled or unlabeled merozoites dissolved in SDS-PAGE sample buffer were loaded in each lane of an SDS–10 to 20% gradient polyacrylamide gel with a thickness of 0.75 mm, or 60 μl of immunoprecipitated antigen was loaded in each lane of a 1.5-mm 10 to 20% gradient gel. Resolved biotin-labeled proteins were electroblotted to NC, while unlabeled proteins were electrotransferred to a polyvinylidene difluoride membrane (PVDF; NEN Research Products, Boston, Mass.). The NC blots were developed as described for immunoblotting, but the antibody step was omitted. The PVDF blots were stained with Coomassie blue.

Trypsin digestion. Approximately 2×10^7 freshly harvested merozoites were washed twice in RPMI 1640 and then resuspended in 2 ml of trypsin-EDTA (BioWhittaker) at 37°C. Three hundred microliters of a digest was removed at 1 and 5 min after digestion. The digests were mixed with 1.2 ml of prechilled FCS to stop the trypsin reaction and centrifuged at 4,000 × *g* for 5 min at 4°C. After three washes with prechilled 50 mM Tris-HCl buffer (pH 7.6), intact merozoites were counted and dissolved in SDS-PAGE sample buffer. An extract of 6×10^4 merozoites/μl was prepared for immunoblotting.

Statistical analysis. PROC GLM of SAS was used to analyze the data (SAS Institute Inc., Cary, N.C.). The *F* test and least significant difference procedure were used to compare the mean values of assay results. A *P* value of <0.05 was taken as a significant difference.

RESULTS

Immunoblot band patterns. The four immunoblot band patterns based on combinations of the Sn30, -16, -14, and -11 proteins (30, 16, 14, and 11 kDa, respectively) are presented (Fig. 1). Nineteen serum and four CSF samples selected from 220 clinical samples were grouped according to their band patterns (Fig. 1 and 2): group 1, N1 to N7; group 2, 31 to 34; group 3, 41 to 46; and group 4, 51 to 56. Serum or CSF samples of group 1 were not reactive with Sn16, Sn14, or Sn11; sera from groups 2, 3, and 4 consistently reacted with Sn16, Sn14, and Sn11, respectively.

Correlation of band patterns with inhibitory activities. The results of neutralization assays are presented in Fig. 2. As expected, serum or CSF samples of the same group showed similar neutralizing activities. Serum and CSF samples of groups 2 and 4 showed approximately equal inhibitory activities, while group 3 sera showed the greatest inhibitory activity of the four

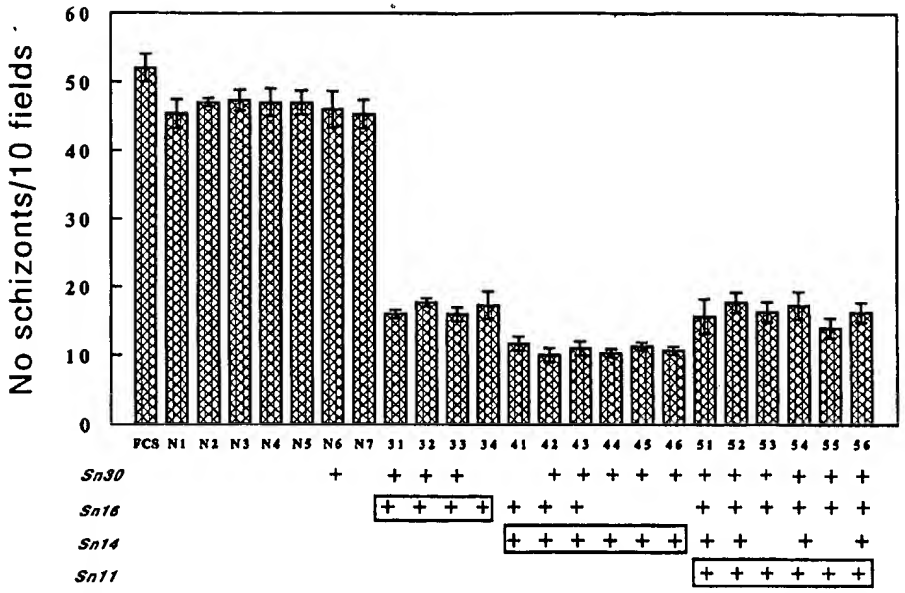

FIG. 2. Neutralization of *S. neurona* infectivity by serum and CSF samples. Merozoites were incubated in serum or CSF samples for 60 min at 37°C. FCS was used as a control. On day 5 postinfection, schizonts in 10 fields (400×) were counted for each flask. Means and standard deviations are presented. Bands recognized by both serum and CSF are shown at the bottom.

groups. Based on these observations, it appears that Sn16 and Sn14 may have important roles during the initial stage of *S. neurona* infection and that antibody to Sn14 may be more effective in neutralization than antibody to Sn16. No inhibitory activity correlating with antibody to Sn30 was noted. Interestingly, group 4 sera that contained antibody to both Sn14 and Sn11 had neutralizing activity similar to that of group 2, suggesting that Sn11 antibody in serum may block the neutralizing effect of Sn14 antibody (Fig. 1 and 2).

Surface protein labeling and immunoprecipitation. A combination of surface protein labeling, immunoprecipitation, and Western blotting was conducted to determine whether Sn16 and Sn14 are surface proteins. Proteins similar in size to these two proteins were labeled (Fig. 3A, lane b), a result confirmed by immunoprecipitation (Fig. 3A, lane c). Negative serum did not precipitate any *Sarcocystis* protein (Fig. 3A, lane d).

Effect of trypsin digestion. The rapid action of trypsin suggests that these proteins were very accessible to the action of the enzyme and therefore on the cell surface. Within 1 min the Sn14 band was no longer visible, and the Sn16 band showed significantly reduced density at 5 min (Fig. 3B). The density of the Sn30 band was also reduced after 5 min. The trypsin-resistant band between Sn16 and Sn14 in Fig. 3B was recognized by only few equine sera and was apparently not a surface protein, as determined by surface labeling and immunoprecipitation (Fig. 3A). Since trypsin digestion could lyse the parasite, digestion was monitored by counting intact merozoite cells. A significant reduction in merozoite numbers was not observed until after trypsin digestion for 40 min.

DISCUSSION

Humoral immunity may play an essential role in clearing *S. neurona* merozoites at the extracellular stage. Specific antibodies may lyse the merozoites via complement, inhibit their infection by blocking attachment and penetration, or bind to surface receptors and disorder signal transductions. These results suggest that sera containing antibodies specific for Sn16 and Sn14 reduce parasite infection, probably by binding to the merozoite cell surface and blocking attachment and/or penetration. An extensive body of data is available to indicate that antibody to apicomplexan parasites is protective. Treatment of *Cryptosporidium*-infected immunocompromised patients with hyperimmune bovine colostrum has ameliorated or eliminated clinical symptoms (38), an effect correlated with antibodies specific for sporozoite surface proteins (12, 29, 36). Invasion of

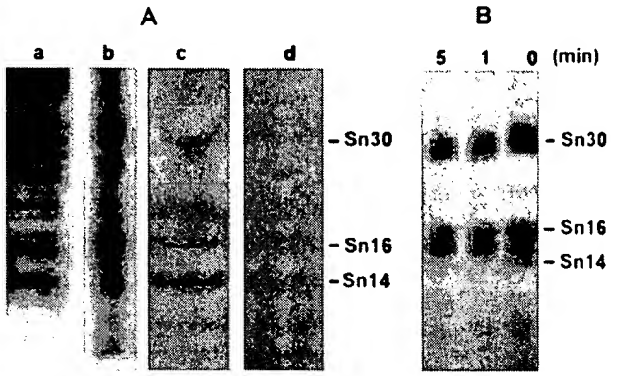

FIG. 3. Evidence for surface localization of Sn14 and Sn16. (A) Analysis of surface proteins by labeling, immunoprecipitation, and Western blotting. Lane a, proteins in an extract of unlabeled merozoites following SDS-PAGE and electrotransfer to PVDF (Coomassie blue stain). Lane b, proteins in an extract of biotin-labeled merozoites following SDS-PAGE and electrotransfer to NC. The blot was probed with peroxidase-streptavidin conjugate. Lane c, biotin-labeled proteins in a lysate of biotin-labeled merozoites immunoprecipitated with serum from a horse with a histologically confirmed case of EPM. Precipitated proteins were separated by SDS-PAGE and electrotransferred to NC. The blot was developed as described for lane b. Lane d, band pattern of biotin-labeled proteins in a lysate of biotin-labeled merozoites immunoprecipitated with EPM-negative serum and developed as described for lane c. (B) Trypsin digestion. Merozoites were digested by trypsin for 1 and 5 min at 37°C followed by SDS-PAGE and electrotransfer to NC. The blot was treated with serum from a horse with a histologically confirmed case of EPM.

target cells by trypomastigotes of *Trypanosoma cruzi* is receptor mediated and can be blocked by specific antibodies (1, 39). Yet another example is the penetration-enhancing factor of *Toxoplasma gondii* that has been identified by using monoclonal antibodies (34).

Detection of *S. neurona* infection by demonstration of reactivity of serum and CSF samples with the Sn11, Sn14, and Sn16 antigens has been extensively used as a diagnostic tool (5, 6, 32) and is sensitive (20, 21). However, the test has not yet been fully validated by studies of serum and CSF samples from cases in which *S. neurona*-like organisms have been detected histologically and cultured. Neutralization assays revealing significant differences in inhibitory activities between the groups of serum and CSF samples with different immunoblot band patterns strongly suggest that antibodies specific for Sn14 and Sn16 have protective activity against *S. neurona*, at least in vitro (Fig. 1 and 2) and support the use of the immunoblot test in diagnosis of EPM. Antibodies to Sn30 are not recognized as specific since a 30-kDa antigen immunoreactive with sera from horses with EPM is found in other *Sarcocystis* spp.

The serum neutralization data obtained in this study were based mainly on the use of an in vitro bioassay developed in our laboratory. Although assays for other apicomplexan parasites such as *Cryptosporidium* (12), *Plasmodium* (24), and *Toxoplasma* (27) species have been described, this study represents the first application of such an assay to a *Sarcocystis* sp. Optimum inhibition required sensitization of merozoites in serum or CSF for at least 40 min (data not shown), suggesting that maximum inhibition of parasite infection requires saturation with specific antibody. Although serum and CSF samples of the same band pattern group did not have equal antibody activities as estimated by immunoblotting (Fig. 1), all samples saturated their antigens under the assay conditions and gave similar inhibitions in neutralization assays (Fig. 2). This result was supported by serum dilution and time of incubation data (data not shown). Although schizont and merozoite counts under conditions of maximum inhibition were not equal for these two experiments, percent reductions in numbers of schizonts and merozoites were very similar, i.e., 84 and 92% in the serum dilution assay, compared with 81 and 84% in the incubation assay. These small differences in counts resulted from the unequal dosages of merozoites used for infection in the two assays. Reductions in schizont production by group 3 sera ranged from 78% (sample 45) to 80% (sample 42) relative to the FCS control (Fig. 2). These highly consistent results suggest that the assay was valid and that counts of schizonts and merozoites can serve as indicators of inhibitory activity.

Although *S. neurona* was sensitive to specific antibodies, a 10-min exposure to antiserum was required to yield a significant reduction in parasite production (data not shown). This may partially explain why protective antibodies to some apicomplexan parasites are effective in vitro but not in vivo (23). Newly released parasites are exposed to serum for a shorter time in vivo, and the access of neutralization-sensitive epitopes to antibody may be limited (31). Merozoites in vivo may move more directly from cell to cell. However, in the case of EPM, disease occurs only after the merozoite passes through the vascular endothelium of the blood-brain barrier into the central nervous system, and so humoral responses may play an essential role in blocking this migration. Moreover, specific cytotoxic T cells are ineffective in attacking merozoites migrating to the central nerve system in the bloodstream.

When antibodies to Sn11 and Sn14 (group 4) coexisted, the inhibition activity of the serum was reduced to that of sera of group 2 (Fig. 2), suggesting that antibody to Sn11 blocked the effect of Sn14 antibody. Therefore, these two proteins may be located in close proximity on the merozoite surface.

The results of biotin labeling and immunoprecipitation studies (Fig. 3A) are consistent with the hypothesis that the effects of antibodies to Sn14 and Sn16 are mediated via binding to surface antigens. A combination of these techniques has been shown to be effective in the identification of specific surface antigens (8). However, since nonantigenic proteins may be coprecipitated, the results may not be definitive. The results of controlled trypsin digestion were, however, consistent with the conclusion that Sn14 and Sn16 are localized on the surface of the parasite (Fig. 3).

Although monoclonal antibodies are often used to study parasitic proteins, the sera of naturally infected animals have unique advantages in that they can provide important information on protectively immunogenic proteins in the natural host. The parasite may express different proteins at different stages of in vivo or in vitro development; and some proteins may be expressed and function essentially only in vitro. Such proteins would be inappropriate targets for vaccine development. *S. neurona* infection of the horse induces production of antibodies to Sn16 and Sn14, indicating that these two proteins are expressed in vivo and are strong immunogens in the horse. Clearly, they warrant further investigation as candidate antigens for inclusion in vaccines against *S. neurona* infection.

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Immunization of Cattle with Recombinant *Babesia bovis* Merozoite Surface Antigen-1

STEPHEN A. HINES,[1]* GUY H. PALMER,[1] DOUGLAS P. JASMER,[1] WILL L. GOFF,[2] AND TERRY F. McELWAIN[1]

Department of Veterinary Microbiology and Pathology, Washington State University,[1] and Animal Disease Research Unit, Agricultural Research Service, U.S. Department of Agriculture,[2] Pullman, Washington

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Cattle immunized with a recombinant merozoite surface antigen-1 molecule (MSA-1) produced high-titered antibody that reacted with the surface of the parasite and neutralized merozoite infectivity in vitro. However, recombinant MSA-1 immunization did not confer protection against challenge with virulent *Babesia bovis*. These results indicate that antibody-mediated neutralization of merozoite infectivity in vitro, at least for MSA-1-specific antibody, does not reflect in vivo protective immunity to babesiosis.

Cattle that survive infection with blood stages of *Babesia bovis* develop immune responses that protect against subsequent challenge (19). The mechanisms of immune protection to hemoparasite blood stages are incompletely understood (5, 7, 35, 38). However, classical passive transfer experiments demonstrate clearly that antibody can play a role in the prevention of bovine babesiosis (17). These antibodies presumably bind to critical, surface-accessible epitopes of sporozoites, merozoites, and/or infected erythrocytes, thereby blocking essential processes or mediating opsonization (18, 35). Merozoite surface antigen-1 (MSA-1) is an immunodominant 42-kDa *B. bovis* integral membrane glycoprotein that is distributed diffusely across the merozoite surface (6, 11, 12). We have previously shown that monospecific antiserum to native MSA-1 neutralizes the infectivity of merozoites for bovine erythrocytes in vitro (12). In the work reported here, we have tested the hypothesis that the induction of neutralizing antibody to MSA-1 with a recombinant molecule will protect cattle against a virulent challenge.

Cloning and expression of MSA-1 in *Escherichia coli* has been previously described (12). The MSA-1 cDNA insert in pBluescript (pBv42) encodes a 45-kDa fusion protein, of which 3.9 kDa is vector-encoded polypeptide. pBv42 also lacks a typical signal sequence. To determine if the recombinant protein (rMSA-1) encoded by pBv42 is missing a significant amino-terminal sequence in addition to the signal peptide, the DNA sequence 5′ to the cDNA clone was determined, using a genomic clone. Briefly, *B. bovis* genomic DNA was partially digested with *Sau*3A and ligated to *Bam*HI-digested λDASH II vector DNA (Stratagene, La Jolla, Calif.). The genomic library was screened for plaques that hybridized with a previously described ^{32}P-labeled RNA probe that is specific for the 5′ end of MSA-1 (12). A 6.7-kb *Bam*HI DNA fragment containing the MSA-1 gene was excised from a positive lambda phage and subcloned into the unique *Bam*HI site of pBluescript. Dideoxy chain termination sequencing (28) primed by an oligonucleotide primer corresponding to bases 41 to 67 of the original cDNA clone extended the 5′ sequence an additional 207 bases (GenBank accession number M77192). The coding sequence was confirmed by sequencing the corresponding region of *B. bovis* total RNA with reverse transcriptase and a ^{32}P-end-labeled antisense primer also derived from bases 41 to 67 of the cDNA sequence (13).

An ATG start codon at position 108 of the genomic sequence was in frame with the previous cDNA sequence, while stop codons were present in the other two possible reading frames. A predicted TATA box was present at position 17 (31). Translation of the completed 5′ sequence revealed that the pBv42 cDNA clone was missing the sequence for 34 amino-terminal amino acids: MATFALFISALCCVLAITSA * GEEL TQSDVRNADT. There were a predicted signal sequence (underlined) and a cleavage site (*) between amino acids 20 and 21 (37). Therefore, if the signal sequence is cleaved in *B. bovis* as predicted, the rMSA-1 protein encoded by pBv42 is missing only 14 amino acids of the native polypeptide.

rMSA-1 was immunoaffinity purified from bacteria containing pBv42, using monoclonal antibody BABB35A4 coupled to Sepharose 4B (Pharmacia) (21). Briefly, a bacterial lysate was prepared by previously described methods (12) and tested for the presence of rMSA-1 by Western blotting (immunoblotting; not shown). Unlike its native counterpart, which is a hydrophobic glycoprotein (11), rMSA-1 is soluble in phosphate-buffered saline (PBS). This difference in hydrophobicity probably reflects the inability of the prokaryotic vector to modify the translated protein by attachment of the glycosyl phosphatidylinositol anchor moiety present on the native protein (9, 11). Soluble antigen was passed through the BABB35A4-Sepharose 4B column. Antigen eluted with potassium isothiocyanate was examined for purity by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24). The purified recombinant fusion protein migrated as a doublet at an apparent molecular weight of 43,000 to 45,000 and reacted with monoclonal antibody to MSA-1 in immunoblots (not shown).

Fifteen 6-month-old Holstein calves were randomly divided into two groups of ten and five animals. Each of the 10 animals in group A was immunized three times at 2-week intervals by intramuscular injection of 100 μg of purified rMSA-1 mixed with 6 mg of saponin in PBS. A fourth immunization 4 weeks later consisted of 200 μg of purified protein in saponin-PBS. At each time point the five control animals in group B received adjuvant alone (saponin in PBS). Serum was collected 2 weeks following the last immunization to test for antibody titers and in vitro neutralization.

Isotype-specific antibody to MSA-1 was measured by an

* Corresponding author. Mailing address: Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6069. Fax: 335-8529. Electronic mail address: shines@vetmed.wsu.edu.

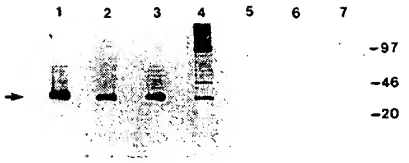

FIG. 1. Immunoblot demonstrating specificity of the antibody response in immunized cattle. Sera from animals immunized with rMSA-1 (lanes 1 to 3) recognized native MSA-1 (arrow), while sera from adjuvant-treated controls (lanes 5 to 7) did not recognize *B. bovis* antigen. Postinfection, hyperimmune serum from an animal challenged four times with blood stages of *B. bovis* (B101) recognized multiple proteins in the same antigen preparation (lane 4). All sera were diluted 1:1,000. Molecular weight (in thousands) markers are indicated at right.

enzyme-linked immunosorbent assay in which affinity-purified rMSA-1 was bound directly to polystyrene plates (19a). Briefly, antigen-antibody complexes containing bovine immunoglobulin G subclass 1 (IgG1) or IgG2 were specifically detected with monoclonal antibody DAS-17 or DAS-2 (courtesy of Allen Guidry, U.S. Department of Agriculture, Beltsville, Md.), respectively, and then with horseradish peroxidase-conjugated goat anti-mouse IgG and 2.2′-azino-di[3-ethyl-benzthiazoline sulfonate (6)] substrate. A positive reaction was an optical density reading at 450 nm that was 3 standard deviations above the mean value of negative sera from the five adjuvant control animals. The titer recorded was the reciprocal of the highest serum dilution giving a positive reaction. To demonstrate that bovine antibody to rMSA-1 recognized parasite-derived protein, sera were also tested for the ability to bind Texas-T_2Bo strain merozoites by fixed immunofluorescence (20, 23). The monospecificity of the antibody response was examined by immunoblot, using *B. bovis* parasite antigen (approximately 2.5 $\times$ 10^7 infected erythrocytes per lane) (11, 30).

All 10 animals immunized with rMSA-1 (group A) produced antibody to merozoite antigen that was detected by immunofluorescence (mean titer, 1,350) and was specific for *B. bovis* MSA-1 in immunoblots (Fig. 1). None of the group B adjuvant control animals developed detectable antibody titers. Cattle receiving rMSA-1 developed both IgG1 and IgG2 isotype antibodies to rMSA-1. However, the IgG1 titers were 3.3- to 100-fold higher (Table 1).

Neutralizing antibody to MSA-1 was detected by using a previously described in vitro assay (12). Briefly, serum was heat inactivated (56°C for 30 min), diluted 1:5 in complete culture medium, and incubated with 5 $\times$ 10^5 merozoites (T_2Bo strain) for 30 min at 4°C. Merozoites were then used to initiate microaerophilous stationary-phase cultures (16) in 96-well plates by adding fresh, uninfected bovine erythrocytes. All sera were tested in triplicate. After 96 h of culture, the percentage of parasitized erythrocytes was determined by light microscopy of Giemsa-stained slides prepared from each well. Serum containing antibody to rMSA-1 effectively neutralized the infectivity of Texas strain merozoites in vitro (Table 1) (mean percent parasitized erythrocytes, 0.34%) compared with serum from adjuvant controls (mean percent parasitized erythrocytes, 6.33%; $P < 0.001$ by Student's t test).

Having demonstrated the ability of rMSA-1-specific antibodies to block merozoite invasion and survival in vitro, we tested the hypothesis that these immune responses would protect cattle in vivo. At 3 weeks following the last immunization, all 15 cattle were challenged intravenously by subinoculation of 1.8 $\times$ 10^3 infected erythrocytes (T_2Bo strain) from an infected, splenectomized calf. This dose was shown by previous titration experiments to represent a virulent challenge. Starting 5 days

TABLE 1. Antibody responses to immunization with rMSA-1

Immunogen and animal no.	Antibody titer to MSA-1		In vitro neutralization (% parasitized erythrocytes)[a]
	IgG1	IgG2	
rMSA-1			
B679	1,000	100	1.27 (0.52)
B680	10,000	100	1.27 (0.88)
B681	1,000	300	0.11 (0.03)
B682	3,000	300	0.05 (0.04)
B684	10,000	300	0.05 (0.04)
B686	300	30	0.21 (0.14)
B687	10,000	300	0.14 (0.04)
B689	3,000	100	0.14 (0.07)
B691	3,000	100	0.07 (0.06)
B693	10,000	100	0.09 (0.05)
Mean			0.34[b] (0.47)
Adjuvant[c]			
B694			6.50 (0.65)
B695			7.53 (0.82)
B697			7.90 (0.28)
B698			5.83 (1.4)
B699			3.87 (2.7)
Mean			6.33 (0.88)

[a] Mean (standard deviation) at 96 h.
[b] $P < 0.001$ versus adjuvant-treated control by unpaired Student's t test.
[c] Adjuvant-immunized cattle were used to establish mean background optical density level (at 450 nm) at each dilution tested.

before challenge, cattle were monitored daily for fever, changes in packed cell volume, and the presence of parasites in Giemsa-stained blood smears. Despite the induction of neutralizing antibody to MSA-1, cattle in group A were not protected against bovine babesiosis compared with group B adjuvant-treated controls (Table 2). There were no significant differences in the percent decrease in packed cell volume, the lowest packed cell volume, the days of detectable parasitemia, or the number of days in which the rectal temperature exceeded 103°F (39.4°C) between MSA-1 vaccinates and controls. It is clear from these experiments that rMSA-1, when used as a single antigen and in a format that induces high titers of antibody, is not an appropriate immunogen for the prevention of bovine babesiosis.

Although there is strong evidence that antibody is important in the immune control of *B. bovis* infection, this study suggests that antigens other than MSA-1, such as those found on the surface of infected erythrocytes or in apical organelles, are the targets of those protective responses (2, 27, 32, 34). These results also raise questions regarding the role of neutralizing antibody in immunity. The ability of monospecific serum to MSA-1 to neutralize merozoite infectivity in vitro could reflect conditions which are not present in vivo. Merozoites are ex-

TABLE 2. rMSA-1 immunization trial: response to virulent challenge

Immunogen (n)	Mean (standard deviation)		
	% Decrease in packed cell vol	Lowest packed cell vol	Days of detectable PPE[a]
rMSA-1 (10)	57.9 (15.8)	15.4 (7.0)	3.3 (1.3)
Adjuvant (5)	64.5 (7.7)	13 (3.3)	1.8 (2.8)

[a] PPE, percent parasitized erythrocytes.

posed to serum for shorter periods of time in vivo, and accessibility of neutralization-sensitive epitopes to antibody binding may be limited (29). Likewise, merozoites in vivo may move more directly from cell to cell, especially in the spleen. However, these experiments do not exclude the possibility that in vitro neutralization will correlate with in vivo protection for other surface molecules which are more accessible due to distribution (e.g., apical proteins) or have a different role in host cell invasion.

The failure of anti-rMSA-1 antibody to protect cattle could also reflect a failure to induce sufficient antibody of the protective isotype (4). Isotypes of bovine IgG differ in biological activity, especially in their ability to interact with Fc receptors on phagocytes (22). The immunization protocol used here induced a predominant IgG1 response (Table 1). If opsonization by antibody is important for in vivo protection (3), it may be necessary to immunize with a method that specifically stimulates an IgG2 response (22). Further, if T_H subset bias in cattle is similar to that in mice, antibody isotype may be profoundly influenced by the T_H subset response (8, 25). The relation of T_H response to immunity against bovine babesiosis is currently unknown but is under active investigation (7, 36). An immunization method that more effectively targets the protective T_H phenotype could produce dramatically different results (1).

MSA-1 is encoded by members of a multigene family that also encodes MSA-2, a 44-kDa glycoprotein that is coexpressed on the merozoite surface (12, 14). Simultaneous expression of a related but antigenically variable surface molecule could provide a mechanism for invasion of host erythrocytes by an MSA-1-independent pathway (10). Accordingly, an immunogen that targets one molecule or one pathway may not be sufficient. In malaria, immunization of mice with cells expressing the circumsporozoite gene of *Plasmodium yoelii* resulted in only partial protection against challenge, while addition of cells expressing a second sporozoite surface protein protected mice completely (15). In future trials, it may be important to include parasite proteins, such as MSA-2 and those from *B. bovis* apical organelles, which play critical roles in host cell invasion and are less polymorphic (26, 32–34).

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Monoclonal IgG antibody-mediated protection against *Sarcocystis neurona* infection

Hany M. Elsheikha[1], Alice J. Murphy[2], Jon S. Patterson[2], Mary G. Rossano[1], and Linda S. Mansfield[1,3]*

[1]Department of Large Animal Clinical Sciences, [2]Diagnostic Center for Population and Animal Health, [3]Department of Microbiology and Molecular Genetics, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA

Abstract

In order to understand immune correlates of protection and to develop effective immunization strategies against EPM disease, it is important to know if antibodies can confer protection against *Sarcocystis neurona* infection. This study was conducted to test the hypothesis that IgG antibodies confer protection against *S. neurona* infection. A panel of IgG monoclonal antibodies (MAbs) was evaluated in tissue culture systems and by using Gamma-Interferon-gene-Knockout (IFN-$^{\gamma}$-KO) mice. Each of the MAbs tested formed immuno-fluorescence on the entire surface of *S. neurona* merozoites to at least a titer of 1:1250 and recognized 16 and 30KDa bands by Western-blotting. Each MAb diluted 1:25 in culture media reduced the attachment and invasion of *S. neurona* merozoites into equine dermal (ED) cells compared to controls. A mixture of all MAbs diluted to 1:25 or 1:50 in culture media almost completely inhibited attachment and invasion. Additionally, intraperitoneal injection of IFN-$^{\gamma}$-KO mice with the MAbs one day before and the day of challenge and on day 1 and 2-post challenge conferred a considerable level of protection against infection with sporocysts of *S. neurona* delivered orally. This MAbs treatment prolonged the mean time to death (MTD) of immunized compared to control groups of mice. Passive transfer of the MAbs before the time of challenge and later showed no adverse or toxic effects on either the mice or the ED cultured cells. We conclude that the IgG response can impede the establishment of infective *S. neurona* parasites in the mouse intestine and these MAbs are candidate molecules for immunotherapy of *S. neurona* infection in horses. The potential for use of individual antigens as protective immunogens in preventing *S. neurona* infection is raised

Key words: *Sarcocystis neurona*; IgG; monoclonal antibody; passive immunization; protection.

Introduction

Sarcocystis neurona has become recognized as an important causative agent of equine protozoal myeloencephalitis (EPM) (Dubey et al., 1991). EPM is the most common neurologic disease in horses in the Americas (Dubey et al., 2001). An estimated 60 to 89.3 % of horses are seropositive (Beth et al., 2003; Rossano et al., 2003). In addition to being a cause of serious discomfort to horses, EPM also has been associated with adverse neurological function and can be often fatal (Mackay et al., 1997). Recent information indicates that EPM should be taken more seriously, not only because of its increasing prevalence but also because of its devastating economic impact on the horse industry in the United States (NAHMS, 1998; 2000). In spite of research efforts to develop therapeutic agents and vaccines against the causative agent of EPM, effective control remains elusive. No commercially available anti-parasitic chemotherapy is consistently effective in treating this disease. Likewise, the current vaccine strategy focuses on targeting the whole parasite antigen resulting in variable efficacy probably because immune serum produced varied greatly in the composition, isotype, and specificity of microbe-binding antibodies. In contrast, monoclonal antibodies (MAbs) provide a homogenous preparation with which to investigate the variables that contribute to antibody-mediated protection. Therefore, MAbs might be a potential alternative immunotherapy against *S. neurona* infection.

In the last decade, it has become increasingly evident that multiple independent mechanisms are involved in the development of *S. neurona* infection, depending on host and parasite factors, or a combination of both. However, the cellular mechanisms of pathogenesis of *S. neurona* are not well defined. Moreover, the significance of the antibody response to *S. neurona* infection is not clearly defined except for a few studies (Witonsky et al., 2004). The life cycle of *S. neurona* in horses is complex and involves asexual development within the horse neural tissues "aberrant intermediate host" as well as sexual development within the gut of the definitive host, the opossum. Infection of horses occurs via ingestion of food or water contaminated with the sporocyst stage of the parasite, followed by sporocyst excystation and liberation of sporozoites in the horse small intestine. This is followed by the crucial step of sporozoite invasion of horse gut epithelial cells into the blood stream, which is a key step prior to successful development and establishment of the infection in the neural tissues. For this reason, the interaction between parasite and host cells at this site plays a critical role as the first host

defense barrier and may provide promising molecular targets for vaccine intervention. Additionally, the merozoites remain in the extracellular phase till reach to the neural tissue during which they are susceptible to humoral immunity.

The role of antibody in protection against a microorganism is often studied directly, by passive antibody transfer in a relevant animal model. Our hypothesis was to test the potential for intraperitoneal inoculation of a mixture of MAbs to conferr protective immunity to *S. neurona* infection in IFN-$^{\gamma}$-KO mice. Using this mouse model, we have tested a panel of MAbs to determine the possible role of IgG antibody for humoral immunity to *S. neurona* infection. Our data showed that passive transfer of IgG MAbs can confer remarkable protection against exposure to *S. neurona* infection in tissue culture system and if parasite infection occurs in IFN-$^{\gamma}$-KO mice, their presence can ameliorate the onset and subsequent pathogenic manifestations of *S. neurona* infection even without synergistc T-cell help.

Materials and methods

Animals, animal room, and housing

Seven-to eight-week old inbred specific-pathogen-free female mice were used for the protection experiment. These mice (C.129S7 (B6)-*Ifng*^{tm1ts}) lack the functional gamma interferon gene (IFN-$^{\gamma}$-knockout) and have been widely used as a model for pathogenesis and infectivity studies of *Sarcocystis neurona*. Mice were obtained from a commercial breeder (Jackson Laboratories, Bar Harbor, Maine, USA). All described procedures were approved by the Institutional Animal Care and Use Committee and conducted in compliance with accepted standards of the *Guideline for the Care and Use of Laboratory Animals* (NIH Publication 86-23, revised 1996). The mice had been monitored by the vendor and found free of the following pathogens; *Klebsiella* species, cilia-associated respiratory bacillus, *Pasteurella pneumotropica*, *Staphylococcus aureus*, *Bordetella bronchiseptca*, *Corynebacterium kutscheri*, *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, *Mycoplasma* species, *Citrobacter rodentium*, *Pseudomonas aeruginosa*, *Salmonella* species, *Clostridium piliforme*, *Helicobacter* species, *pneumocystis carnii*, ectromelia virus, mouse hepatitis virus, mouse parvovirus, mouse rotavirus, mouse encephalomyelitis virus, K virus, Hantaan virus, lactate dehydrogenase-elevating virus, mouse minute virus, pneumonia virus of mice, sendani virus, lymphocytic choriomenengitis virus, polyoma virus, thymic virus, mouse adenovirus, mouse cytomegalovirus. Also, mice were

examined every day for signs of illness and monitored by periodic tape tests for ectoparasites and fecal flotation for endoparasites and results were negative.

Mice were maintained and received humane care in a specific pathogen-free (P-2 level) animal facility at the Michigan State University Laboratory Animal Containment Facility. Animals were housed, three to five mice per cage, in autoclaved 11.5x7.5x5-in. polycarbonate cages with stainless steel lids and covers fitted with a 0.22-μm filter (micro-Isolator™, Lab Products, Inc., Maywood, N.J.). Maple hardwood chip bedding (Harlan Sani-Chips, Harlan TeKlad, Madison, wis.) was changed twice weekly. Animals were maintained on a laminar flow bench with controlled temperature (22±2°C), humidity (60± 10%), and lighting (12-h light/dark cycle). A commercially formulated irridiated mouse diet (7913; Harlan TEKIAD, Madison, wis.) and acidified water (pH 2.5 to 3.0) were offered *ad libitum*. The floor of the animal room was cleaned by use of a vacuum cleaner, and the racks of the laminar flow benches and the floor were disinfected with a 50 ppm solution of sodium hypochlorite. Mice were acclimatized for at least one week prior to being used in the experiment.

Parasite and tissue cultures

For the in vitro experiments, *S. neurona* isolate MIH1 was used. Merozoites of this strain were cultured from neural tissue of an EPM diseased horse from Michigan, USA in 1997, and thereafter maintained in our laboratory in equine dermal (ED) cell cultures (Mansfield et al., 2001). ED cells; the cell line used throughout this study were purchased from American Type Culture Collection (ATCC, CRL-1390) and used between passages 25-29. Cells were routinely grown in 75-cm^2 cell culture flasks in 15-20 ml of Dulbecco modified Eagle's medium (DMEM) supplemented with 10 mM HEPES buffer, L-glutamine, 10,000 IU/ml penicillin G sodium, 10,000 μg/ml Amikacin, 250 μg/ml Amphotericin B, nonessential amino acids, 100 mM sodium pyruvate, and 10% heat inactivated fetal bovine serum (FBS) (GIBCO Invitrogen Corp., Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO_2/95% air until the monolayer became confluent when the medium was changed to maintenance medium (6% FBS). This medium supported both host cells and parasites. The medium was changed once a week. ED cells were passaged once a week.

For the in vivo experiment, *S. neurona* sporocysts were originally isolated from an

opossum gut in 2002 and purified by centrifugation of the mucosal homogenate on potassium bromide discontinuous density gradient centrifugation as described (Elsheikha et al., 2003). Sporocysts were genotyped using the diagnostic 25/396 DNA marker as described (Tanhauser et al., 1999). In March 2003, ~ 1,000 of these sporocysts induced neurological signs and encephalitis in an inoculated IFN-$^{\gamma}$-knockout mouse 33 days post inoculation (DPI). One week prior to mice inoculation, sporocyst viability was assessed by propidium iodide (PI) exclusion assay as described (Elsheikha and Mansfield, 2004a).

Monoclonal antibodies

We need to write some information about the source and nature of these Mabs obtained from IDEXX Company.

Evaluation of MAb Cytotoxicity

ED cells were dislodged from a confluent ED monolayer in a 75-cm^2 flask by 0.25% trypsin dissociation. Cell viability was tested by trypan blue dye exclusion assay and then plated in 24-well cell culture plates (Cat. No. 3526, Costar, Corning Inc., Corning, NY, USA) at ~ 2 X 10^3 cells per well and grown on 10% DMEM until confluent. Media was removed and cells were incubated with about 50 µl of the MAb (1:25 dilution in DMEM) for the same mount of time (1.5 hr) the merozoites were exposed to the MAb in the in vitro parasite inhibition experiment. Cells fed medium without MAb treatment were used as controls. One ml 6% FBS DMEM was added to each well after 1.5 hr. After 24 hours, media was removed and replaced with fresh 6% FBS media.

Qualitative evaluation

MAb-treated and control cells were examined at 0 hr, 4hr, 8hr, 24hr, 48hr, and 96hrs, post treatment. The cell monolayers were inspected visually first at 200x magnification using an inverted microscope (Nikon, Olympus-CK2, Japan) for any signs of cell lyses such as the presence of rounded cells, detached cells, and/or granules. Then cells were examined at 400x magnification for the presence of intracytoplasmic vacuoles indicative of cytotoxicity. Experiments were run in triplicate.

Quantitative evaluation

In addition to microscopic observation, we used a quantitative assay to assess cytotoxicity of the MAbs. This was a non-radioactive cell proliferation technique known as the Methylene blue staining assay. This assay is based on the release of methylene blue from stained ED host cells, as reported for fibroblast cells (Oliver et al., 1989). Culture media was removed from the wells and cells were washed 3 times briefly with 1 ml of 0.12 M saline, and excess fluid was removed. The cell monolayers were then fixed by adding 1 ml of a 10% formaldehyde saline solution and the plate was incubated at room temperature (RT) for 30 min. The formaldehyde solution was removed into a separate hazardous waste container. One ml of filtered 1% (w/v) methylene blue stain was added and the plate was incubated for 30 min at RT. The dye was removed and wells were washed 4 times with 0.01 M 1x borate buffer (pH, 8.5). The cell bound dye was released by addition of 1 ml of elution solvent solution 1:1 (v/v) ethanol and 0.1 M HCl. The remaining cell monolayer stained with methylene blue and was checked microscopically. One ml of the eluted fluid per well was distributed into 10 wells of a 96-well plate. The absorbance of the contents of the wells was measured spectrophotometrically at 630 nm in a microplate reader (Bio-Rad) after correcting for background absorbance with wells containing only 100 µl of the elution solvent. This experiment was performed at 1, 2, and 4-days post treatment. Data were expressed as mean absorbance values (optical density) derived from 10 replicate samples in two separate experiments.

In vitro inhibition of parasite growth

ED cells were subcultured in 6-well plates (Cat. no. 3516, Costar) seeded with ~8×10^3 cells per well. For experimentation, cell monolayers were grown overnight or until confluent at 37°C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS. Prior to inoculation of plate wells with the parasite, merozoites were incubated in triplicate in a medium containing MAb at 37°C for 1.5 hr. Positive and negative assay controls were untreated merozoites and culture media, respectively. Plates were incubated in a sterile condition at 37°C in a 5% CO_2 atmosphere. Media was changed at 24hr and then weekly thereafter. In the first experiment, each MAb was tested individually at a dilution of 1:25 in 6% FBS DMEM. The numbers of plaques were

counted in all wells at 3 and 4-weeks post infection. In a second experiment, a mixture of all the MAb at dilution of 1:25 each and 1:50 each in 6% FBS DMEM were tested in triplicate as per individual MAb procedures. The numbers of plaques were counted in all wells at 3, 4, and 5-week post infection. Plaques were defined as areas where cells were coming loose surrounded by rounded up cells associated with the presence of merozoites and schizonts. In the first experiment, culture media containing merozoites were centrifuged for 10 min at 1,200 rpm to pellet the whole cells and large particles. Supernatants were removed and centrifuged for 30 min at 1,200 rpm. The pellets were then resuspended in 1.5-ml 6% DMEM which was aliquted into the required numbers of tubes. In the second experiment, *S. neurona* merozoites used were first separated from host cells using PD-10 column as described (Elsheikha et al., 2004b).

Measurement of Mabs Activity

Immunofluorescence assay (IFA)

For preparation of IFA slides, merozoites were harvested and washed with 1x phosphate buffered saline (PBS), and resuspended in PBS. Serial dilutions of the merozoite stock were placed in duplicate in wells of 12 well slides (20µl/well), air dried and fixed in acetone. Cerebrospinal fluid (CSF) from a horse previously tested positive for *S. neurona* antibodies was placed on each well and incubated for 30 min at 37°C. The slide was washed several times with PBS and then incubated with Fluorescein isothiocyanate- (FITC)- labeled goat anti-horse IgG- h+1 secondary antibodies in PBS and 0.1% Evans blue and incubated at 37°C for an additional 30 min. The slide was washed with PBS, cover-slipped and the optimum dilution of merozoites for IFA slides determined. The stock merozoite solution was diluted with PBS to the optimum and multiple slides prepared through the fixation step and stored frozen at -20°C until used.

Monoclonal antibodies (6A, 6B, 9A, 11A, 17A, 19B) were applied at 1:2500 and 1:10,000 dilutions in PBS and incubated for 30 min at 37°C followed by washing as above. Serum from a knockout mouse previously infected with *S. neurona* was used as a positive control. FITC-labeled goat-anti-mouse IgG-h+1 secondary antibody (Kirkegaard & Perry Labs, Inc., Gaithersburg, MD, USA) diluted 1:10 in PBS and 0.1% Evans blue dye (as a general protein counterstain) were applied. Stained merozoites were observed by epifluorescent

microscopy using the SPOT RT slider "F" mount camera (model No. 2.3.1, Diagnostic Instruments Inc, Sterling Heights, MI, USA) and SPOT RT software V3.3. Pictures were taken using laser scanning microscope (LSM Zeiss axioskop 2 MOT, Jena, Germany) equipped with Zeiss LSM 5 Pascal Confocal Unit and LSM software version 3.0.

SDS-PAGE and western blotting

Parasite antigens and pre-stained protein molecular weight standards (Life Technologies, Inc., Gaithersburg, MD, USA) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using 12-18% continuous gradient gels under reducing conditions, and transferred on nitrocellulose membranes (Millipore). To block non-specific binding sites, the membrane was incubated for 60 min with Tween Tris Buffered Saline (TTBS) with Bovine Serum Albumin (BSA). Blots were incubated with (dil 1:10) primary MAbs in 1% BSA in Tween Tris (hydroxymethyl) aminomethane Buffered saline (B-TTBS) overnight. Then, blots were 3 x washed with TTBS, followed by 4 hr incubation with biolabelled goat antibody to mouse IgG (dil 1:10) antibody conjugated to HRP (KPL). To assess non-specific reactions, control strips were similarly prepared, but without using the primary antibody. Protein binding was detected by incubation of the blot for 45 min with ExtrAvidin peroxidase conjugate (Sigma) 1 µl/ml TTBS.

Preparation and injection of MAbs into mice

Six anti-*S. neurona* IgG MAbs (6A, 6B, 9A, 11A, 17A, 19B) were combined and evaluated for their prophylactic effect against a *S. neurona* sporocyst challenge in seven-to eight-week old IFN-$^{\gamma}$-KO mice. The rationale for selection of MAb combinations was based on preliminary data obtained from in vitro MAb binding inhibition assays. The ascites of 6A, 6B, 9A, 11A, 17A, and 19B were prepared for injection. A mixture of these antibodies was prepared from equal volumes of the ascitis fluids by diluting the fluids in sterile PBS such that each antibody will have a final dilution of 1:50. MAbs were administered intraperitoneally by injection of 0.5 ml of ascitis dilution. In the first experiment, mice were divided into 4 groups each of 3 mice as follow: (i) Group of 3 mice were gavaged with 500 sporocysts in 0.2 ml PBS per mouse intragastrically by using a rubber French catheter. One day prior to challenge and the day of challenge, and on the

first and second day post-challenge mice received combinations of ascites containing the six IgG MAbs by i.p. injection, (ii) Group of 3 mice were gavaged with 500 sporocysts and 0.5 ml of sterile 1x PBS I.P. and used as a positive control, (iii) Group of 3 mice were gavaged with 0.2 ml PBS and received 0.5 ml MAb ascites I.P. and served as a control for any potential side effect of the antibodies, (iv) Group of 3 mice were gavaged with 0.2 ml PBS and received 0.5 ml of sterile 1x PBS I.P and served as a negative control. This experiment was repeated again in a second trial using the same design used in the first experiment with the following two modifications: (1) the number of mice was increased to 5 mice per group and (2) the inoculation dose was lowered to 100 viable sporocysts per mouse.

Documentation of *S. neurona* infection in mice

Mice were monitored every day for the development of any clinical signs. As soon as mice showed any clinical signs of illness, they were euthanized by CO_2 inhalation. To determine if the mice that survived the challenge possessed the same parasite burden in their brains and other body organs, all remaining mice and control mice were killed at the same time the positive control, untreated mice were killed. Specimens from lung, intestine, liver, spleen, kidney, heart, and muscles were collected, fixed in 10% neutral buffered formalin, and processed for histopathology and immunohistochemistry. Also, the brains were halved by sagittal section, and small pieces of forebrain, midbrain, and cerebellum from each of the halves were examined by immunohistochemistry and IFA. The presence of schizonts or merozoites was scored histologically as previously described (Dubey and Lindsay, 1998).

Portions of the brain were used for extraction of the parasite DNA for confirmation of the infection by PCR and sequence analyses. The brain of each mouse was assayed for the presence of the schizont and merozoite stages of the parasite by inoculation into confluent monolayer of ED cell cultures in T25-cm flasks of brain homogenate in DMEM as described (Dubey and Lindsay, 1998). All inoculated culture flasks were monitored daily for the appearance of any signs of parasite growth and plaque formation under a light inverted microscope (Carl Zeiss, Opton, Columbia, MD, USA).

To test for the potential for excretion of sporocysts in mice feces following challenge with *S. neurona* sporocysts, feces from the animal bedding were collected daily for the first 3 days post inoculation and examined for the presence of sporocysts using the sucrose fecal

floatation technique, Diamant-Fuchsin stain, and PCR-sequence analyses as described (Elsheikha et al., 2004a).

Statistics

Results were reported as means plus or minus standard errors of the mean. Differences between groups (MAb-treatment and controls) in all experiments were initially explored for significance using the Fisher's Exact Test. The Fisher exact probability test is a non-parametric technique for analyzing discrete data when the two independent samples are small in size. It is used when the scores from two independent random samples all fall into one or the other of two mutually exclusive classes, in this study either death or survival of mice. This analysis was followed by examination by one-way analysis of variance (ANOVA). Calculations were performed using commercially available statistical software (SAS PC Version 8, SAS Institute Inc., Cary, North Carolina). A P value less than 0·05 denoted statistical significance.

Results

MAbs toxicity on cultured host cells

Following MAbs treatment, the in vitro culture system used allowed us to study any specific cytologic changes to ED cells as well as to quantify any cytotoxicity to the cells. Toxic effects of the MAb on cultured ED cells were evaluated by using microscopic observation. The viability of cell preparations was determined before the experiments using trypan blue dye exclusion assay and was found to be ~99%. In one plate, 2 duplicate wells were incubated for 90 min with the MAbs mixture, and then fresh media was added. In 2 wells of another plate, cells were not exposed to MAbs. In both cases, wells were inspected every four hr over a course of 96 hr to assess viability, integrity, and to examine any toxic effects of MAbs on the cells compared to control untreated cells. The condition of cells throughout the incubation period was monitored by Nikon phase-contrast microscopy. No significant difference was noticed between MAbs treated and untreated ED cells. MAbs used in this study did not cause any cytopatheic lesions in ED cells compared to controls. The cytotoxic effect of MAbs was also evaluated quantitatively using the methylene blue staining assay. This assay was used to determine if the MAbs had any toxic

effect on the host cells, which in turn would inhibit their metabolic activity and thus diminish their ability to multiply. This assay assesses the viability of the cells by comparing the numbers of viable-MAbs treated host cells to viable-untreated host cells. Cells treated with MAbs showed no measurable cytotoxicity over the course of 96 hr (Fig. 1).

MAbs inhibitory effect on *S. neurona* merozoites in vitro

Prior to in vivo passive transfer studies with MAbs, we studied the in vitro inhibitory characteristics of these antibodies alone, and in combination, against *S. neurona* infection in ED cell monolayers. Evaluation of the inhibitory effect of MAbs on parasite infectivity in tissue culture relied on microscopic observation of the host cell monolayers following infection with the parasites. The nature and extent of host cell damage were assessed using an inverted phase-contrast microscope and was scored for each treatment as the mean number of plaques created by replicating merozoites (±s.e.m). For experiment 1, ED cells were cultured to confluence in 6-well culture plates. *S. neurona* parasites (~ 4×10^3/well) were added to the confluent ED monolayers. At the same time, ED monolayers in separate 6-well plates were incubated with the same parasite concentration after incubation with separate MAb for 1.5 hr. There were 3 replicates of each for this experiment. Data were recorded at 3, 4, and 5 weeks post inoculation. In experiment 2, ED cell monolayers were inoculated with *S. neurona* parasites (4×10^3/well). At the same time ED monolayers in a separate 6-well plate were incubated with the same parasite concentration after incubation with a mixture of MAbs at 2 different dilutions (1:25 and 1:50) for 1.5 hr. There were 3 replicates each for this experiment. Data were recorded at 3 and 4 weeks post inoculation. All MAbs used in the in vitro studies conferred a high degree of protection against *S. neurona* infection and significantly reduced mean plaque scores compared to those of control ($P < 0.05$). From a functional perspective, the most intriguing observation is that the MAbs used in this research either individually (Fig. 2A) or in combination (Fig. 2B) were all capable of inhibiting the parasite invasion to cultured cells. Parasites treated with MAbs produced lesser numbers of plaques than untreated parasites, which underwent unrestricted proliferation leading to substantial cell disruption.

MAbs recognizes distinct parasite proteins by Western immunoblotting and IFA assays

A reproducible banding pattern was observed for parasite lysates in western immunoblotting. MAbs reacted largely against an antigenic fraction with a mol. Wt. of at 30,000, although it also had some slight reactivity against a 16,000 mol. Wt. antigen. MAbs to *S. neurona* cell membrane antigens was measured with the IFA assay utilizing *S. neurona* cultured-derived merozoites. IFA demonstrated antibody binding to the *S. neurona* merozoite surface.

Prophylactic effect of IgG MAbs on *S. neurona* infection in mice

To determine whether MAbs could protect naïve IFN-$^{\gamma}$-KO mice against *S. neurona* infection, passive immunization studies were carried out. The quantity of antibodies used in protection studies was based on a previous determination of the amount of antibody required to be reactive in the IFA assay. A combination of MAbs administered prophylactically to mice delayed the onset and severity of infection in treated mice compared to controls. In the first experiment, infected, untreated mice developed clinical signs at 14 DPI, 2 days earlier than the infected and treated mice. Also, clinical signs were less intense in treated mice compared to the untreated mice. However, at 25, 26, and 27 DPI one mouse of each group were euthanized due to the progression of the disease. In the second experiment, infected and untreated mice developed clinical signs at 13 DPI, 6 days earlier than the infected and treated mice. On 29 DPI all the five infected and untreated mice were euthanised due to progession of severe clinical signs. In contrast, one out of five of the infected and treated mice was euthanised at 30 DPI because of a severe head tilit, one mouse was found dead 33 DPI and the remaining mice were euthanised at the same day, 33 DPI due to progression of clinical signs. All control mice whether mock-inoculated with PBS or MAbs did not develop any clinical signs throughout the whole experiments.

Detection of *S. neurona* sporocysts in mice feces

Fecal flotation and Diamant-Fuchsin staining assays did not detect any *S. neurona* sporocysts in any mice feces from all different mice groups. However, PCR using LSM1 and LSM2 primers produce a PCR product from DNA extracted from feces collected from cage of MAb-treated mouse group only.

Discussion

The present study was conducted to test the ability of specific monoclonal antibodies to protect IFN-$^{\gamma}$-KO mice from a challenge with a *S. neurona* infection. The results demonstrated that MAbs to *S. neurona* major surface antigens delayed the onset and severity of *S. neurona* infection in IFN-$^{\gamma}$-KO mice, thus providing support for the role of the surface antigens in active immunity and the potential use of these proteins as protective immunogens.

Although cell-mediated immunity appears to be the major component of the host's defense mechanism against intracellular organisms, antibody-based immunity, particularly serum IgG antibodies, play at least some part in the host's response against many intracellular pathogens (Robbins et al., 1995). Studies with monoclonal antibodies have demonstrated passive protection for several microbes where experiments with polyclonal immune serum had provided negative or inconsistent results, including *Candida albicans* (Han and Cutler, 1995), *Cryptococcus neoformans* (Dromer et al., 1987), *Listeria monocytogenes* (Edelson et al., 1999), *Leishmania mexicana* (Anderson et al., 1983), and *T. gondii* (Sayles et al., 2000). For these pathogens, the identification of protective monoclonal antibodies established the precedent that antibody could be effective and dispelled the notion that humoral immunity was ineffective due to an inherent limitation in the activity of this arm of the immune system.

In this study, microscopic examination of ED cells allowed us to study the cytotoxicity of MAbs. Likewise, the methylene blue assay allowed us to assess the host cell damage by MAbs treatment in a convenient, easy procedure. Oliver et al. (1989) described the methylene blue staining assay for use on rat and human fibroblast and lung cells. The assay is quick, inexpensive, and particularly applicable to adhered cells. The proliferation assay is based on the cellular staining properties of methylene blue, which can then be released from the cell into solution by lowering the pH, and then quantitated by measuring absorbance. This method was very effective for measuring cell numbers because it measures stain incorporation by all the cells in the well, thereby eliminating cell-to-cell field variation that occurs in a solid phase immunohistochemical methodology and standard counting.

On the basis of the number of detectable antigens in the western blot analyses, 30KDa and 16KDa appear to be the most predominant targets of the humoral response to the parasite. The utility of MAbs in the control of *S. neurona* infection was tested by passive immunotherapy

studies with MAbs in IFN-$^{\gamma}$-knockout mouse model. Results presented here demonstrate that IgG MAbs can reduce infection by *S. neurona* parasite. While cellular immunity is required to overcome *S. neurona* infection immunocompetent hosts, IgG directed to neutralization-sensitive merozoite epitopes may have utility in passive immunization against *S. neurona* infection. The six IgG MAbs selected recognized surface P30 on merozoites and have been shown to decrease infection levels in vitro and in the mouse model, indicating that P30 appears to have an essential role in the invasion process of the parasite and contains neutralization-sensitive epitopes. Because P30 is conserved among geographically diverse *S. neurona* isolates (Elsheikha and Mansfield, 2004b), present in both infectious sporozoite and merozoite stages, and might contain neutralization-sensitive epitopes, it may be a biologically relevant antigen, which can be targeted for immunological intervention. Pretreatment of *S. neurona* merozoites with MAbs abolished the adhesion and invasion of parasites to cell monolayers. These findings suggest the presence of contact-dependent inhibitory effects (i.e., involvement of carbohydrate-containing molecules in these processes) of the MAbs used in the present study. Results of the present study support the relevance of P30 and suggest that IgG targeted to this antigen may be a functional immune response to *S. neurona* infection. The in vitro and in vivo procedures as reported here provide a model system for studying the pathogenicity of *S. neurona* in detail. The knowledge of *S. neurona*-host cell interactions will provide insight into the mechanisms of host cytopathogenicity and the pathobiochemistry of *S. neurona* infection.

The incomplete efficacy of passive MAbs against *S. neurona* in KO mice particularly in those mice gavaged with 500 viable sporocysts suggests that antibody-mediated protection might be independent of T cells and implied that other mechanism may be operative and high levels of inhibitory antibodies are required to achieve complete protection. This suggest that vaccine-induced inhibitory antibody, along with cellular immunity, could offer protection against *S. neurona* infection. Given our knowledge that IgG is one important component of protection, it is reasonable to continue to focus resources aimed at the design of *S. neurona* vaccine immunogens that will elicit potent inhibitory antibodies to the parasite. While we do not know if KO mice model accurately predict the precise quantitative levels of antibody needed to protect horses, the data begin to spot the light on the type of antibody response that could play a role in protection against EPM disease in horses.

In addition to the amount of antibody, immunoglobulin-related variables such as antibody

specificity, isotype, and idiotype can have profound effects on antibody protective efficacy. However, host-related variables can also determine the outcome of passive protection experiments. For example, the protective efficacy of passive antibody to *Salmonella enterica* serovar *Typhimurium* is dependent on the mouse strain used (Eisenstein et al., 1984). For some pathogens, the efficacy of passive antibody is dependent on the presence of intact cellular immunity (Yuan et al., 1997). Also, antibody efficacy can depend on the microbial strain used despite the presence of the target antigen (Mukherjee et al., 1995). The exact number of genotypes of *S. neurona* that occur in nature is unknown. To date we have identified two different genotypes (designated H1 and H2) among 5 *S. neurona* isolates (Elsheikha and Mansfield, 2004b). We have not found mixed infections in naturally infected horses and do not yet know whether cross-reactive immunity plays a role in nature. Nevertheless, epidemiological questions such as these are of clear interest for vaccine development and are currently being investigated.

In summary, these initial studies have shown that MAbs can bind to the merozoite stage of the parasite life cycle and significantly inhibit infection in vitro. Additionally, passive immunization of IFN-$^{\gamma}$-KO mice with these MAbs suggests that the immunodominant surface antigens are partially protective antigens and as such are primary candidate for use in a subunit vaccine against the *S. neurona* infection. These findings point the way toward their testing in horse trials for immunotherapy of *S. neurona* infection in horses.

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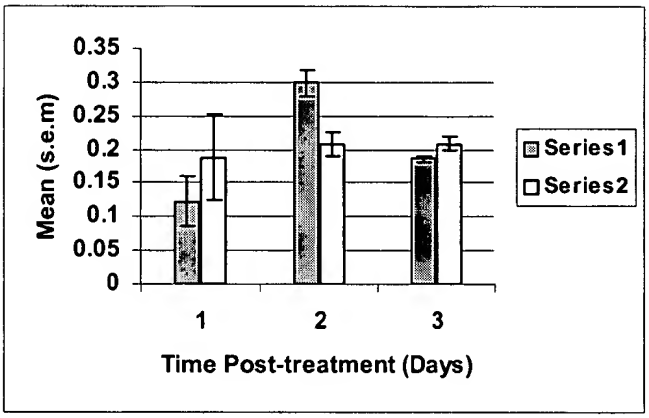
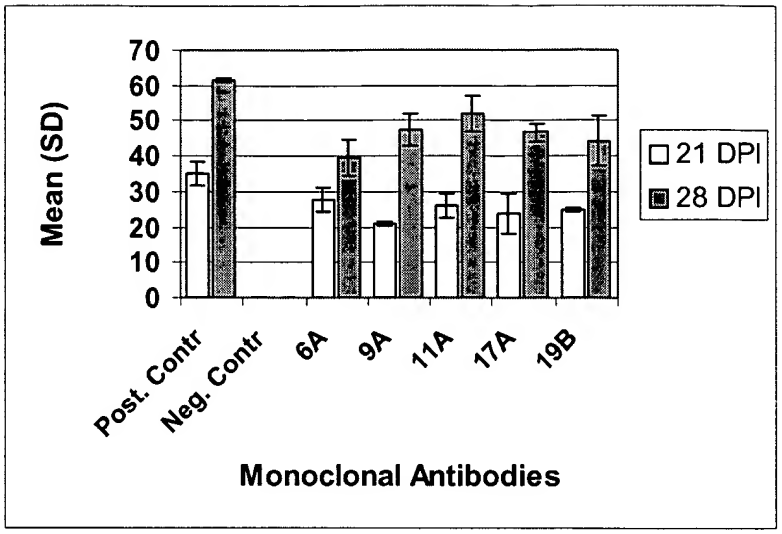


Figure 1. Time course of cytotoxicity of equine dermal cell monolayers by mixture of monoclonal antibodies (■) compared to control untreated cells (□). Cytotoxicity was determined by methylene blue assay as described in the text.



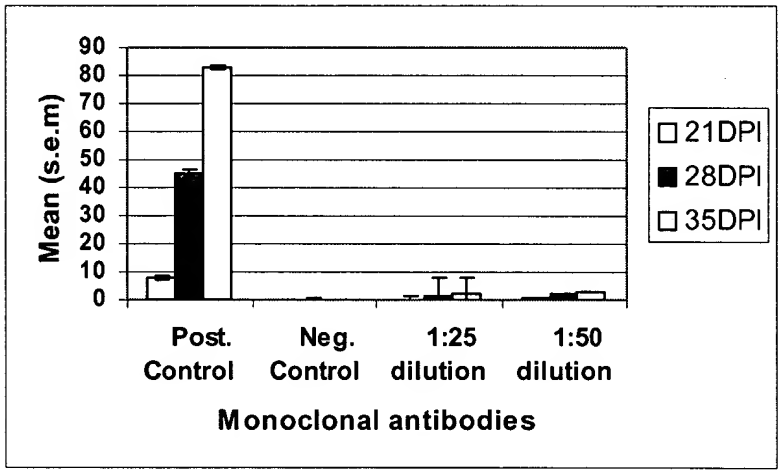


Figure 2. Inhibitory effect of monoclonal antibodies (MAb) against infection with *Sarcocystis neurona* merozoites in equine dermal cell cultures. (A) using individual MAb. (B) using MABs combination

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TRANSMITTAL OF APPEAL BRIEF (Small Entity)

Docket No. MSU 4.1-526

In Re Application Of: **Linda S. Mansfield, Mary G. Rossano, Alice J. Murphy and Ruth A. Vrable**

Application No.	Filing Date	Examiner	Customer No.	Group Art Unit	Confirmation No.
09/670,096	09/26/00	Padmavathi Baskar	21036	1645	7494

Invention: **VACCINE TO CONTROL EQUINE PROTOZOAL MYELOENCEPHALITIS IN HORSES**

COMMISSIONER FOR PATENTS:

Transmitted herewith in triplicate is the Appeal Brief in this application, with respect to the Notice of Appeal filed on: June 28, 2004

☒ Applicant claims small entity status. See 37 CFR 1.27

The fee for filing this Appeal Brief is: **$165.00**

☒ A check in the amount of the fee is enclosed.

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

☒ The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **13-0610**

☐ Payment by credit card. Form PTO-2038 is attached.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

Signature

Dated: **08/20/04**

Ian C. McLeod
Registration No. 20,931
McLeod & Moyne, P.C.
2190 Commons Parkway
Okemos, Michigan 48864
Phone No. (517) 347-4100
Customer No. 21036

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to "Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450" 08/20/04 (Date)

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